Felix Bronner
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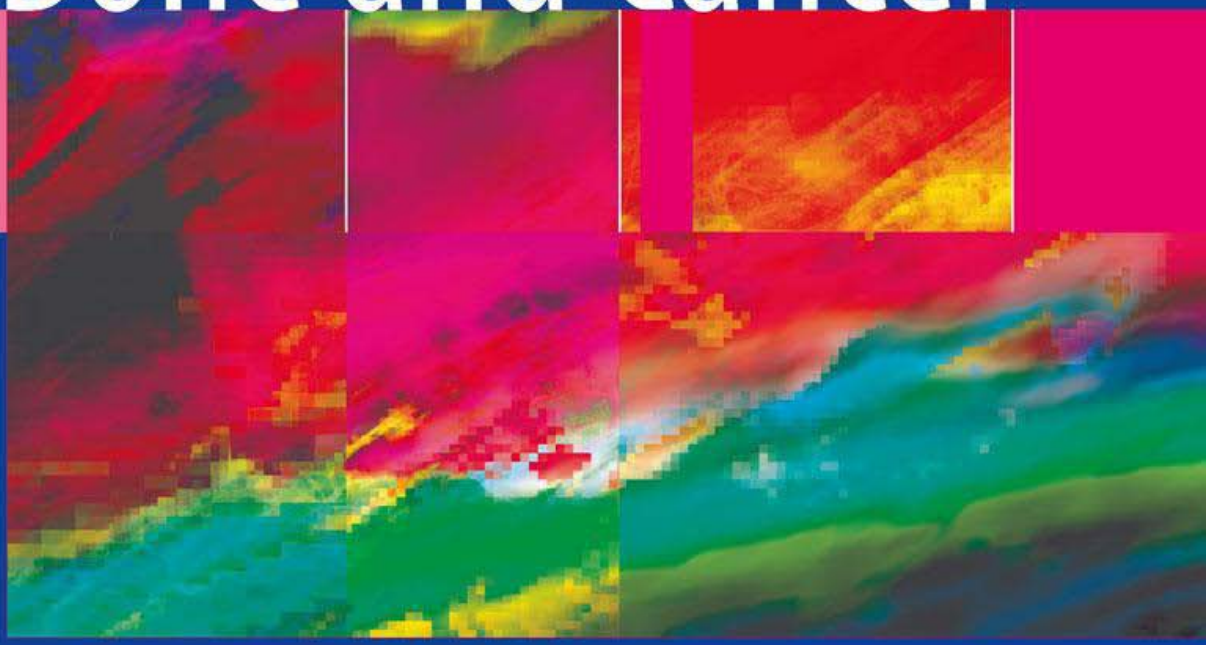
Bone and Cancer



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Series Editors

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Bone and Cancer

Springer

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Preface

Skeletal cancers may originate in bone as primary tumors, or arrive there as a consequence of the metastatic dissemination of cancer cells from distant sites, giving rise to secondary or tertiary tumors. Progression of malignant cancers in bone is fostered by the multipotential bone marrow stromal cells and the complex cellular milieu to which they give rise, including osteoblasts, osteoclasts, hematopoietic stem cells, bone marrow endothelial cells, and their precursors. Changes in genotype and phenotype enhance tumorigenicity of cancer cells in bone, as they adapt to and remodel the bone marrow microenvironment. Because cultured bone cells that have been in contact with cancer cells for extended time periods can transform non-tumorigenic cells, it is apparent that bone cells undergo phenotypic and genotypic alterations during skeletal cancer progression. Components of the complex bone microenvironment contribute significantly to the growth and proliferation not only of primary cancers, such as osteosarcoma or myeloma, but also to the process of metastasis of epithelial-derived cancers such as prostate or breast cancer.

Bone and Cancer, as have previous volumes of *Topics in Bone Biology*, deals with the basic science, translational, and clinical aspects of bone and, in this case, the relationship to cancer. Written by authorities, the chapters discuss background and history, proceeding to the questions of the day, with emphasis on what yet is to be learned. The material is of interest to medical, dental and graduate students, resident physicians and dentists, bone researchers, and all those concerned with understanding how bone attracts and becomes home to so many cancers.

Aaron M. Havens, Yusuke Shiozawa, and Russell S. Taichman, in Chapter 1, discuss in detail the relationship between hematopoiesis and the bone marrow niche in which hematopoietic stem cells and early hematopoietic progenitor cells differentiate. The niche integrates changes in nutrients, oxygen, and in paracrine and autocrine signals that in turn alter the rate of cell multiplication. The niche is also the site to which metastasizing cancer cells are attracted and where they multiply. The chapter discusses in cellular and molecular terms how osteoblast surfaces, which effectively create a niche, play a role in hematopoiesis. Discussion then proceeds to vascular and marrow niches, the still largely unknown role of adipocytes, of reticular cells, and to the concept of cells homing to bone during fetal life and in many cancers.

The genetics of osteosarcoma, a relatively rare primary bone tumor, are discussed by Marc F. Hansen in Chapter 2. After describing the histopathology of osteosarcoma, Hansen describes unconventional subtypes, as well as the more common sarcoma of head and neck. He then proceeds to analyze the inherited predisposition to osteosarcoma. The Li–Fraumeni syndrome is discussed, as is the Rothmund–Thompson syndrome, and the relationship to Paget's disease. The remainder of the chapter is devoted to an analysis of the genetics of osteosarcoma, along with a detailed discussion of the role of genes like RB1, TP53, the role of the Wnt signaling pathway, genomic stability, and chromosomal instability. Like most chapters, Chapter 2 is extensively referenced.

In Chapter 3, Rajesh Sehgal, Kristen M. Sanfilippo, and G. David Roodman discuss multiple myeloma, the most common hematologic malignancy in adults. After describing the pathophysiology of bone disease in multiple myeloma, the authors discuss the role of osteoclast activation and of RANKL and MIP-1α in increasing osteclastogenesis, of PTHrP as the major mediator of hypercalcemia, and of IL-6 acting to stimulate osteoclast formation and of IL-7, IL-3, and DKK1 in inhibiting osteoblast activity, differentiation, and preosteoblasts. The chapter then describes the clinical manifestations of myeloma, including bone destruction, hypercalcemia, neurologic, and other systemic complications. Diagnosis, prognosis, and treatment are evaluated. The chapter also discusses Hodgkin's disease, non-Hodgkin's lymphoma, and adult cell leukemia/lymphoma, and their involvement in bone. This chapter, like many, has pertinent illustrations.

The role of the bone marrow endothelium in cancer metastasis is discussed in Chapter 4, by Carlton R. Cooper, Robert A. Satcher, Lisa A. Gurski, and Kenneth L. van Golen. Bone pain, pathologic fractures, spinal cord compression – termed skeletal-related events – are the result of cancer cells metastasizing to bone, a process brought about by bone marrow endothelial cells that promote entry into the bone marrow and lead to cancer growth. The authors describe the natural history of bone cancer, its prognosis and clinical course, proceeding to an overview of endothelial cells and their role in bone physiology and tumor angiogenesis. The components of the metastatic phenotype are then discussed, with reference to the Rho GTases, their role in angiogenesis and endothelial cell motility. On the basis of their own findings, the authors conclude that information on one cell type, e.g., HUVEC, cannot be extrapolated to other cell types, e.g., BMEC. Therefore, therapeutic approaches targeting BMEC cannot be based on findings with HUVEC. Several tables enhance the value of this chapter.

The role of lysophosphatidic acid in bone physiology and bone cancer has been clarified only in recent years. Olivier Peyruchaud and Norman J. Karin, in Chapter 5, describe this molecule, its major in vivo source, its biological activities, its receptors, its expression in bone cells, and its rapid effects on bone and cartilage cells. These include calcium signaling, MAP kinase activation, and rearrangements of the cytoskeleton. Long-term effects of lysophosphatidic acid are on cell proliferation, survival, and differentiation. The cytoskeleton is also rearranged so as to permit cell movement to take place. As yet little is known about how lysophosphatidic acid affects gene expression. In fracture healing, lysophosphatidic acid may modulate proliferation and

migration of osteoblast progenitor cells to the fracture gap. Evidence is cited to the effect that this molecule may foster arthritis progression and that its receptor is expressed in thyroid and prostate cancer cells. Lysophosphatidic acid may also play a role in cancer progression, inasmuch as silencing the molecule reduces disease progression.

The important role the bone microenvironment plays in siting metastasizing cancer cells is dealt with in Chapter 6, by Anna Podolanczuk, Bethan Psaila, and David Lyden. They discuss specific tumor growth factors like the vascular endothelial growth factors that modulate angiogenesis, the fibroblast growth factor that upregulates fibronectin expression and which in turn provides an adherence platform for metastasizing cancer cells. Blocking these factors may slow cancer progression and several such pharmaceutical inhibitors are discussed. An important chemokine that governs migration patterns of hematopoietic cells is CXCL12, also important for metastasis, as are angiopoietin and osteopontin, which retain stem cells in the niche. The authors discuss in detail the role of bone marrow-derived cells for supporting tumor cell survival, promoting their dissemination and migration, and their role in building the niche. Genetic regulation and targeting of metastasis is dealt with toward the end of the chapter.

In Chapter 7, Sabine Riethdorf, Volkmar Müller, Catherine Alix-Panabières, and Klaus Pantel provide information on methods for detecting and characterizing disseminated tumor cells in the bone marrow of cancer patients. They list advantages and disadvantages of the various immunocytochemical and molecular assays of these cells and discuss the significance of detecting them in the bone marrow of patients who have no signs of clinical metastasis. The assays therefore may have prognostic value. In addition, characterization of these relatively few disseminated cells may help define the process of early tumor cell dissemination and therefore help identify novel therapeutic targets.

Inna Serganova and Ronald G. Blasberg, in Chapter 8, analyze and discuss molecular imaging of cancer cells in bone. The most widely used imaging modalities are optical fluorescence, bioluminescence, photon and single photon emission tomography, autoradiography, gamma camera, magnetic resonance spectroscopy, diffusion-weighted imaging, ultrasound, and computed tomography. These techniques are described and analyzed. The chapter then proceeds to a discussion of genes encoding receptors, with emphasis on the somatostatin receptors. Imaging of cancer cells in bone by scintigraphy, magnetic resonance imaging, and positron emission tomography is described, as is application to mouse models; the limitations or advantages of each method are evaluated. The chapter concludes by indicating two areas in bone cancer research that need development, namely appropriate animal models and multi-modality imaging strategies.

In Chapter 9, Larry J. Suva, Richard W. Nicholas, and Dana Gaddy discuss the cytokines and chemokines, signaling molecules that link inflammatory responses with cancer development. Tumors develop in a microenvironment that is predominantly managed by inflammatory cells and that plays a critical role for cancer progression. The chapter discusses the relationship between this microenvironment, the cells in that micromilieu, and the cytokines that stimulate metastases to tumor

progression in bone. They include TNF-α, the interleukins (6, 8, 10, 12, 23), (CXCL12/CXCR4), and TGF-β. Their specific targets and whether they act to promote or inhibit a particular process are discussed in detail. The authors conclude by urging the study of the integrated response of cells within the secreted components in the bone marrow microenvironment.

In Chapter 10, Leland W.K. Chung, John A. Petros, and Mary C. Farach-Carson take on the complex subject of osteomimicry that occurs during bone metastasis of many cancers. The authors introduce the concept of osteomimicry and of some unique signaling phenomena that occur during the processes of osteomimicry to create a signaling triad that is associated with poor prognosis. They also describe the plasticity of cancer cells and speculate on the evolutionary significance of cancer cell differentiation. Particular attention is paid to the important role of the cancer-cell-derived receptor activator, NFκB ligand (RANKL), which can increase bone turnover and ultimately facilitate cancer growth and survival in bone. Finally, the authors speculate on how osteomimicry supports signal amplification leading to cancer progression. Prostate cancer osteomimicry in the bone niche is used to illustrate how the understanding of the molecular signaling cascade of ostemimicry may help diagnosis, prediction of progression, and therapy of prostate cancer metastases.

Bone pain is the most common pain of cancer patients. Patrick W. O'Donnell, Nancy M. Luger, and Denis R. Clohisy address this question in Chapter 11, dealing also with the fractures that cancer patients experience as a result of metastatic disease from osteolytic cancers such as myeloma or breast cancer. They discuss bone pain treatment by radiation, radiofrequency tumor ablation, and the use of radiopharmaceuticals. To be able to test therapeutic approaches to bone pain, animal models are needed and discussed, as are various therapeutics and their effect on remodeling. The authors conclude that the mainstay of treatment of bone cancer pain is opioid analgesia, but there is a need for combined therapies that target the multiple mechanisms that drive bone pain.

In Chapter 12, David J. DeGraff, Fayth L. Miles, Ronald R. Gomes, and Robert A. Sikes discuss in detail small animal models for the study of the various cancers in bone. They deal with the four routes of xenograft administration, orthotopic, intracardiac, intravenous, and intrafemoral/intratibial, in each of the animal models of cancer in breast, lung, prostate, and kidney, and in models of multiple myeloma and melanoma. They also take up, where appropriate, transgenic and syngeneic approaches, the SCID mouse model into which human bone tissue is injected, and the occasional spontaneous animal cancer that is a model for a human cancer. In concluding, the chapter calls attention to the need to model metastasis.

Hormonal and bisphosphonate therapies have been used in many bone cancers. Pamela Taxel and Faryal S. Mirza, in Chapter 13, deal in detail with these therapies in prostate and breast cancer. Hypogonadism is a result of treatment with gonadotropin-releasing agonists, estrogen or orchidectomy. Taxel and Mirza deal with the consequences of this situation, as well as treatment of men in their eighties who often are hypogonadic. Hypogonadism constitutes a major risk factor for osteoporosis. It requires treatment that diminishes the rate of bone mass loss and the likelihood of a

pathologic fracture. An interesting consequence of hypogonadism is the simultaneous loss of estrogen, with the result that bone mass loss is further amplified. In their analysis of treating breast cancer, the authors deal with tamoxifen and its effect in lowering osteoporotic fractures, and with the aromatase inhibitors, which cause a significant lowering of estradiol levels in postmenopausal women, therefore raising the rate of bone loss and risk of fractures. They compare treatment with the aromatase inhibitors, anastrozole, letrozole, and exemestane, and with tamoxifen, on a short-term and long-term basis. The chapter then proceeds to a detailed evaluation of bisphosphonates in the treatment of prostate and bone cancer and to an examination of the prevention of bone loss with specific bisphosphonates, including zoledronic acid. The chapter concludes with a call to treat even moderate osteopenia in patients with breast or prostate cancer and, if initially there is no sign of osteopenia, to do periodic tests of bone turnover and bone mineral density to be able to intervene early if bone turnover remains high or bone mineral begins to decrease.

Chapter 13 discusses treatment of bone cancer due to metastasizing prostate or breast cancer. Chapter 14, by Charles J. Schneider and Stephen S. Grubbs, analyzes therapeutic approaches to kidney, thyroid, and lung cancer. Patients with renal cell carcinoma often have multidrug restistance and in the past were treated with immunotherapy. The chapter discusses newer targeted therapies such as monoclonal antibodies against IL-6 or TNF-α, anti-angiogeneic drugs, or Raf kinase inhibitors. In the discussion of thyroid cancer, the authors distinguish between well-differentiated carcinoma, medullary thyroid carcinoma, anaplastic carcinoma, and actual or potential treatment for these conditions. Almost half of lung cancer patients develop bone cancer. The addition of bevacizumab to carboplatin and paclitaxel, approved by the Food and Drug Administration (USA) in 2006, has led to somewhat longer survival for lung cancer patients and available results are evaluated. The chapter also adds to the discussion of available treatment of breast and prostate cancer.

In 1957 Thomas treated two patients with advanced leukemia with infusions of marrow from their identical twins after they had received high-level radiation. This led to the use of allogeneic transplants in combination with radiation. In Chapter 15, Jose Francisco Tomas and Sergio A. Giralt describe and analyze hematopoietic transplants in patients with a genetic disorder, malignancy, or an intrinsic bone marrow disorder. They distinguish between allogeneic, syngeneic, and autologous transplants, describe treatments that involve radiation or chemotherapy, as applied to acute lymphoblastic leukemia, chronic myeloid leukemia, or the myelodysplastic syndrome. The chapter also discusses Hodgkin's and non-Hodgkin's lymphoma, multiple myeloma, and chronic lymphocytic leukemia. Transplant-related complications are evaluated. The chapter concludes that although allogeneic stem cell therapy has cured a variety of hematological malignancies, complications related to graft-vs.-host diseases and disease recurrence remain major barriers.

The spine is the most common site for cancer that localizes in bone. Chapter 16, by Marsha L. Haley, Peter C. Gerszten, and Steven A. Burton, focuses on stereotactic radiosurgery as therapy for metastatic disease of the spine. The authors review the history of external beam radiation ther-

apy, including the evolution of radiosurgery, the amount of dose that can be delivered, with the radiosensitivity of the adjoining soft tissue the limiting factor. Because radiation therapy provides palliation in a significant majority of patients, radiation oncologists are consulted for evaluation of patients and the chapter provides guidance on how to evaluate patients and to devise optimum radiation dosage. Proper dosage depends on accurate localization of the tumor and the chapter discusses how to achieve that and full target immobilization. It also discusses stereotactic radiotherapy, as a compromise between radiotherapy and stereotactic radiosurgery. The chapter concludes by pointing out that radiosurgery has become an interspeciality because orthopedic and neurosurgeons have become members of the radiation oncology team. This in turn has led to advances and greater potential for new technology.

This volume is the outcome of clinicians from several specialities joining with scientists of diverse orientation. It thus illustrates the need and desirability for a multiple and diverse focus on a disease that still is the most common cause of death. We thank the authors for their dedication and their willingness to let their contributions become part of a larger, integrated whole. Our thanks go to Springer, publishers of the series, for their help in assuring quality and in producing a handsome volume. We dedicate this volume to the many victims of what as yet remains a dreaded disease.

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1.

Blood–Bone Axis and Bone Marrow Microenvironment

Aaron M. Havens, Yusuke Shiozawa, and Russell S. Taichman

1.1 Introduction

The function of the bone marrow is to coordinate hematopoiesis, i.e., the continuous production of mature blood cells throughout life. Hematopoiesis often is envisioned as a pyramidal/hierarchical process with the least differentiated cells, i.e., those of greatest maturation potential at the top, hierarchically speaking, and cells that have undergone terminal differentiation at the bottom. The two major lineages of terminally differentiated blood cells are those derived from the myeloid and lymphoid progenitors. Myeloid cells include red blood cells, platelets, and cells that provide cellular immunity, e.g., macrophages and granulocytes. Lymphoid cells comprise T, B, and natural killer cells, and play a major role in coordinating humoral immunity.

Experimental data suggest that the hematopoietic stem cells (HSCs), which are at the apex of the hematopoietic pyramid, differentiate into hematopoietic progenitor cells which in turn can proliferate at a rapid rate or continue differentiating. HSCs also self-replicate giving rise to identical daughter cells. These in turn can enter the progenitor pool. In the adult mouse, HSCs make up approximately 0.0084% $\pm$ 0.0028% of the total bone marrow cells [53].

Humans are estimated to turnover nearly 10^{12} hematopoietic cells daily [62], an output level that must be maintained throughout a person's life. The normal turnover of mature hematopoietic cells ranges from days (granulocytes) to months (red blood cells) to years (as in the case of T and B cells involved in immunologic memory). The demand on HSCs for a constant supply of hematopoietic progenitors is therefore substantial. To be able to meet this demand, HSCs must achieve a balance between differentiation and self-renewal, with cell-intrinsic and -extrinsic factors influencing the decision whether cells self-renew or differentiate. Yet, how the cell decides whether to differentiate or remain a stem cell remains unclear. For survival and reproduction to occur, mechanisms must exist for the integration of and response to information from the environment, including nutrient intake and avoidance of predators, so as to be able to pass on their genome. These requirements are termed the 'ecological niche'. After fetal life, blood cells are produced almost exclusively by the bone marrow. The bone marrow cavity develops as blood precursors migrate and colonize embryonic bone and cartilage. As a

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result bone and blood cells come into close association, but whether this is a stochastic or ordered and directed process has not been resolved yet.

1.2 The HSC Niche in Marrow

In 1978, Ray Schofield used the term 'niche' to describe the physical environment that supports HSCs [108]. The bone marrow niche is a microenvironment in which HSCs proliferate, mature, and give rise to myeloid, erythroid, and lymphoid progenitors. Within the niche, HSCs are believed to receive support and growth signals originating from diverse sources, including fibroblasts, endothelial and reticular cells, adipocytes, and osteoblasts. Signals to HSCs and early hematopoietic progenitors are believed to occur in a structure commonly referred to as the 'niche.' The exact structure of this niche is not known, as antibodies that uniquely define the structure have not been identified, nor are there specific assays that define the niche. It is also unclear what role the HSC niche plays in regulation of other stem cells, cancer stem cells, or other mesenchymal stem cells (MSCs) which are in the niche either by chance or by design. That niche structures somehow regulate growth and survival of HSCs is assured principally through the production of cytokines and intracellular signals that are initiated by cell-to-cell interactions.

The principal function of the niche is to integrate local changes in nutrients, oxygen, paracrine and autocrine signals, and to change HSC quiescence, trafficking, and/or expansion in response to signals from the systemic circulation (Fig. 1.1). How this occurs and the identities of all of the cellular players involved are not known. Moreover, location of the niche itself is

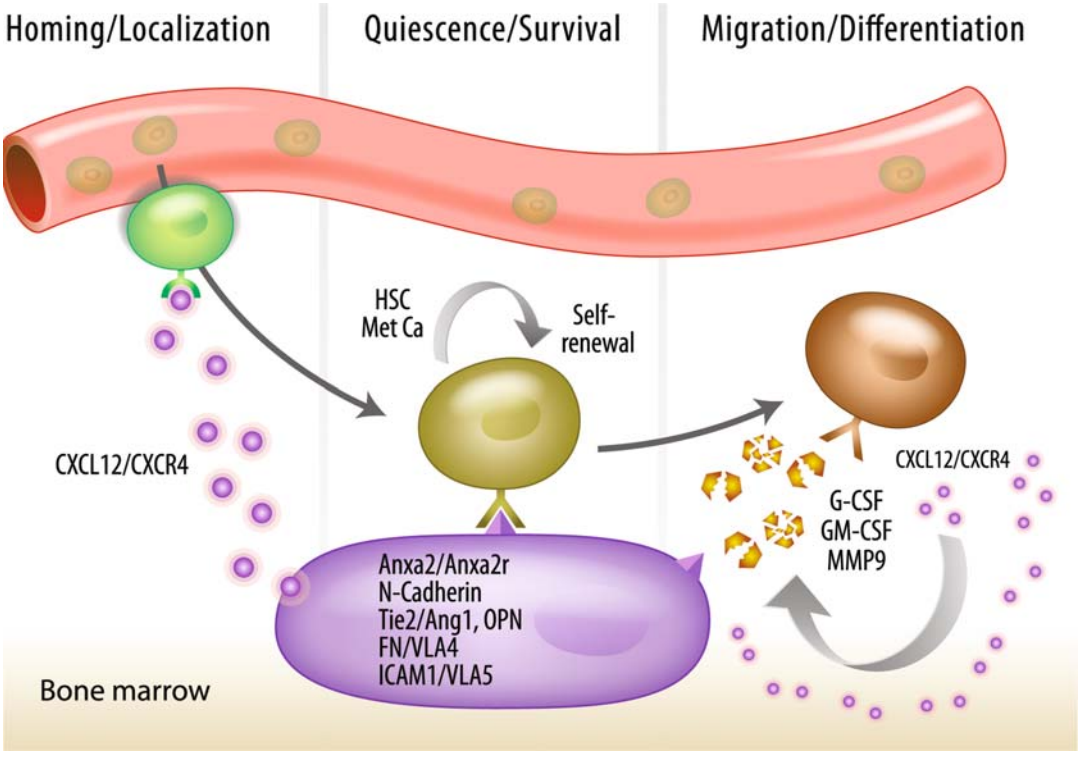

Figure 1.1. Molecular pathways used by stem cells and tumors for homing/localization, quiescence/survival, and migration/differentiation. Significant parallels exist between the use of the endosteal niche by HSCs and tumors. In each case, SDF-1/CXCL12 (labeled CXCL12) and CXCR4 appear to regulate HSC and metastatic cell homing to the niche. Once in the endosteal niche, self-renewal and quiescence is likely maintained by a number of cell–cell and soluble factors that regulate stem cell self-renewal and tumor dormancy. Exit from the marrow is modulated by extracellular cytokines (G-CSF or GM-CSF) and enzyme actions (MMP-9) that cleave off SDF-1/CXCL12.

controversial, including whether it is located close to the endosteal or near the vascular surfaces; if both surfaces are involved, it is uncertain how the functions of the two niches are coordinated.

1.3 Components of the Niche

The most critical cellular components of the niche are probably the HSCs. Identification of stem cells or early lineage cells depends on cell surface markers, that is, antigens associated with states of activation, function, or differentiation. Because morphological identification of early lineage cells is not reliable, it is necessary to be able to identify, in molecular terms, enzymatic and secondary markers of differentiation. Stem cell identification, by definition, relies on the demonstration of the production of a lineage-committed progeny. For HSCs, the identification of CD34, c-kit, and lineage-committed markers (lin^-) has made possible stem cell isolation [28, 56, 63]. Nonetheless, the most effective means to date to distinguish HSCs from progenitor cells is to make use of the relatively simple combinations of SLAM family receptors that are expressed on murine HSCs [63, 136].

From a functional standpoint, HSCs are characterized by their ability to establish multilineage hematopoiesis in lethally irradiated recipients. An in vitro assay to identify stem cells would be highly desirable, but has been difficult to develop: first, because stem cell markers may change in culture and second, because they are linked to the niche. In vitro end points that have been proposed include long-term culture-initiating cells, very-long-term culture-initiating cells, and cobble stone areas [53]. However, in vivo assays currently are the most rigorous and reliable, specifically one using a competitive repopulation assay [37, 38, 47]. In that assay, donor cells must be detectable after 16 weeks, ruling out a possible contribution by hematopoietic progenitors in the graft or host. The presence of multilineage donor-derived cells also must be detectable. If such an assay is used, the full range of hematopoietic reconstitution can be observed even when only single cells are transplanted.

HSCs probably do not rest passively in their niche. A functional dialog likely exists between HSCs and the mesenchymal components of the niche, involving both direct and indirect actions. The functional interdependence between HSCs and mesenchymal cells has been explored from the standpoint of de novo cytokine secretion [120]. When human $CD34^+$ bone marrow cells were co-cultured in direct contact with osteoblasts, IL-6 synthesis was increased by 222 $\pm$ 55% (range 153–288%). During the initial 24-h period the culture of osteoblasts produced nearly 77% of the total IL-6. Direct cell-to-cell contact did not appear to be involved [120]. To our knowledge, these data represent the first demonstration that untransformed cells of the osteoblast lineage can, via the production of a normal bone marrow environment, give rise to normal hematopoietic progenitor cells.

Recently we have found that among the molecules involved in the functional dialog between HSCs and their niche there are several members of the bone morphogenetic (BMP) family that HSCs secrete in response to environmental stresses [49a]. As yet, it is not clear whether the BMP release is a feedback response to alter the HSC pools directly. Several members of the BMP family have been implicated in regulating proliferation, differentiation, and renewal of HSCs [2, 11, 22]. Alternatively, the BMP response may target the mesenchymal population in an effort to increase the size of the niche [49a]. This inference derives support from the finding that mice expressing a conditionally inactivated BMP receptor type IA (BMPrIA) have an enhanced HSC pool, the size of which correlates with the volume of trabecular bone [140]. These findings suggest that HSCs regulate the function and size of their niches. HSCs also seem to be able to direct stromal cell fate and lineage decisions, an activity that heretofore has been attributed to the niche itself.

1.4 Endosteal Niches

Cells that adhere to plastic, many derived from MSCs, support limited hematopoietic activities. Plastic-adherent marrow stromal or bone

marrow stromal cells (BMSCs) are thought to regulate self-renewal, proliferation, and differentiation of the HSCs with the aid of cytokines and intracellular signals initiated by cell-to-cell adhesion interaction [77]. In vitro, BMSCs also promote growth and survival of HSCs and transmit drug resistance to hematopoietic malignancies [10, 69, 101].

As early as the 1970s, studies of the interactions between BMSCs and HSCs had suggested that events localized at endosteal surfaces and, by inference, on osteoblast surfaces played a central role in hematopoiesis [72, 75, 89–91]. Since then, it has been shown that osteoblasts are a crucial component of normal stem cell niches [1, 4, 13, 118, 121, 140, 141] and that HSCs are situated in proximity to endosteal bone surfaces and not randomly distributed in the marrow cavity [32, 44]. These findings are further supported by the observation that HSCs lodge near endosteal surfaces during bone marrow engraftment [85, 98]. Mature osteoblasts are found on endosteal surfaces and share several phenotypic characteristics with the stromal cell lines that support hematopoiesis [24, 61, 83, 119].

Osteoblasts are of mesenchymal origin and are the primary inducers of calcification in the extracellular matrix. In mammals, osteoblast-derived matrices function in support of locomotion, constitute a reservoir for essential minerals, and, by housing them, protect vital organs from injury. Traditionally, bone and blood have been thought to protect the hematopoietic process. Yet, proximity does not necessarily mean that there exists a functional or symbiotic relationship [125]. In the past, osteoblasts were thought to regulate hematopoiesis because osteoblasts induce expansion and maturation of osteoclasts from hematopoietic precursors and activate osteoclastic bone resorption [104, 105]. Consequently, the signals emitted by osteoblasts may act not only on osteoclasts, but also on hematopoietic cells.

Findings from multiple in vivo studies implicate osteoblast involvement in hematopoiesis because they create a niche.Among the earliest in vivo studies are investigations pertaining to the role of the core-binding factor α 1 (Cbfa1) or Runx-2, a transcription factor of the runt-domain gene family. Cbfa1-deficient mice lack both intramembranous and endochondral bone because osteoblasts of mice cannot complete maturation. Homozygous *Runx-2*$^{-/-}$ animals are dwarfed, unable to breathe, and die immediately after birth [25, 66]. The lack of osteoblastic maturation also results in a total lack of bone marrow in the entire skeleton. In these embryos, the number of hematopoietic precursors in the yolk sac is normal at embryonic day E10.5 and normal in the liver at E12.5 [21]. Even as late as E17.5, no significant changes in hematopoietic populations are noted. Just prior to birth, however, the mutant animals develop excessive extramedullary hematopoiesis in the liver and spleen, and large hematopoietic foci in the periportal area. The number of mature granulocytes is increased on E18.5, as is the number of immature hematopoietic cells in the peripheral blood [21]. Mice deficient in the core-binding factor ß (Cbfb, or polyomavirus enhancer binding protein 2^{ß} (PEBP2B)) die around E11.5–E13.5 because fetal liver hematopoiesis does not occur and because of hemorrhage in the central nervous system [107, 132, 133]. Cbfb plays an essential role in Runx-2-dependent skeletal development by enhancing the DNA binding of Runx-2 and Runx-2-dependent transcriptional activation. The mutant animals also have poorly developed marrow cavities [137]. When Cbfb is introduced into *Cbfb*$^{-/-}$ animals driven off from the *GATA1* hematopoietic promoter, the hematopoietic phenotype is largely rescued and the animals survive until birth, but bone formation is severely delayed [137].

Deguchi et al. [21] noted that the extramedullary hematopoiesis phenotype observed in the Runx-2-deficient mice is significantly different from the extramedullary hematopoiesis that occurs secondary to the congenital lack of osteoclasts observed in osteopetrosis in young OP/OP mice [8, 84], c-fos-deficient mice [134], c-src-deficient mice [72], and the double-mutant hck and src mice [73]. Thus, the migration of hematopoietic precursors appears perturbed by a lack of bone marrow cavity in *Runx-2*$^{-/-}$ embryos at a time when the hematopoietic precursors should have migrated to the bone marrow. In comparable fashion, skeleto-hematopoietic perturbations result from alterations in the

condensation/hypertrophy of cartilage precursors. For example, deletion of collagen X or vascular endothelial growth factor (VEGF) leads to a phenotype unable to replace hypertrophic cartilage by normal mechanisms [34, 45]. In the collagen X deletion animals, the unsuccessful establishment of the marrow in bone leads to hematopoietic changes, including altered B- and/or T-cell profiles [34, 45]. Mice also exhibit a similar phenotype in the absence of VEGF [30, 81]. VEGF family members play an essential role in the recruitment of vascular precursors required for blood vessels to be able to invade the endochondral bone anlage and to bring about chondrocyte apoptosis. This in turn is likely to have direct effects on the HSCs [30, 81]. Thus, disruption of VEGF expression or activity not only leads to defects in bone length and to an expansion of the hypertrophic chondrocyte zone, but also interrupts the establishment of a marrow cavity that normally supports the migration of incoming HSCs [30].

Additional findings suggesting that osteoblasts play a vital role in hematopoietic processes are due to the discovery that inactivation of BMPrIA nearly doubles the number of HSCs present in the marrow compared to what is found in the wild-type controls [140]. Co-localization studies have shown that N-cadherin$^+$ osteoblastic cells exhibit similar distribution patterns of HSCs, with N-cadherin asymmetrically localized to the cell surface of HSCs. Calvi et al. [13] have provided comparable data which indicate that overexpressing constitutively active PTH receptors also increases the number of HSCs in the marrow [13]. In this situation, Notch-1 signaling in HSCs matches closely with Jagged-1 receptors on osteoblasts. Moreover, treatment of normal animals with an anabolic regime of PTH also expands the HSC population in the marrow, possibly because of an increase in osteoblast precursors. Puri and Bernstein [97] have shown that neither Tie1 nor Tie2 receptors are required for fetal hematopoiesis, or for the emergence of definitive HSCs, but that both are required for adult hematopoiesis. Arai et al. [4] demonstrated that angiopoietin-1 (Ang-1) expressed by osteoblasts activates Tie2 and promotes tight adhesion of stem cells to their niche. Presumably this adhesion results in HSC quiescence and survival, permitting stem cell maintenance and self-renewal [4] (Fig. 1.1).

A direct approach to defining the role of osteoblasts in development has come from transgenic animals in whom the osteoblast-specific promoter osteocalcin caused diphtheria toxin to be expressed [116]. Later, transgenic mice were developed that had a gene composed of a 1.3-kb fragment of the mouse osteocalcin gene 2 (OG2) promoter so as to express the herpes simplex virus thymidine kinase (Hsv-Tk) gene [18]. In the most definitive set of studies, thymidine kinase expression was put under control of a 2.3-kb type I collagen a1 (Col1a1) promoter fragment [126]. This promoter, like the osteocalcin promoter, is largely restricted to act on differentiated osteoblasts, but is expressed at an earlier point than the osteocalcin promoter used previously [18]. In 8-week-old mice bearing the Col2.3ΔTk transgene treated with ganciclovir (GCV) most osteoblasts had died, bone marrow cellularity was reduced, and the number of osteoclasts was markedly decreased. In the marrow, B cells and erythroid progenitors were reduced as early as 8 days following GCV treatment. These effects were coupled with a progressive loss of stem cells in the bone marrow. This should have led to death from hematological failure. The animals survived, however, because an active extramedullary hematopoietic process had been established [55, 142].

In vitro evidence exists also for an osteoblastic HSC niche role (Fig. 1.1). We have observed (Jung et al., submitted) that soluble signals secreted by HSCs establish a paracrine loop with osteoblasts which is essential for stem cell survival. It seems therefore that HSCs directly modulate the formation of a hematopoietic microenvironment or niche. Moreover, although direct cell-to-cell interactions are not essential for hematopoietic activity, a receptor–ligand interaction between the very late antigen-4 (VLA-4) and the leukocyte function-associated antigen-1 (LFA-1) complexes is necessary. Recombinant ligands such as fibronectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) provide additional engagement or replace the VLA-4/LFA-1 complexes for direct cell-to-cell interactions. The latter induce changes in receptor

levels on osteoblasts of key cell adhesion molecules. Precise details are not known, but N-Cadherin, Wnt signaling pathways, Notch-1/Jagged-1 interactions, and osteopontin and Ang-1/Tie2-mediated events [23] are believed to be important in the establishment of a functional dialog between HSCs and their niche [4, 13, 26, 86, 110, 140]. Furthermore, cell-associated or matrix-bound cytokines including IL-3, Flt3 ligand, granulocyte/monocyte colony stimulating factor (GM-CSF), stem cell factor (SCF), and transforming growth factor-β1 (TGF-β_1) may also contribute to the adhesion events [33, 36, 109, 124].

The precise mechanism as to how HSCs localize to the endosteal surfaces is yet to be delineated. However, the vitronectin receptor ($\alpha_v ß_3$ integrin) on osteoblasts binds to many proteins abundant in bone [42]. LFA-1 and VLA-4 are involved in the adhesion of tumor infiltrating lymphocytes and in osteoclast-to-osteoblast interactions [27, 122]. N-cadherin, Tie2, and Jagged-1 molecules may be crucial to the process that supplements LFA-1/VLA-4 adhesions [4, 13, 140]. Other molecules in the bone marrow also may be involved in the localization of HSCs to the endosteal surfaces. Cell surface studies have shown that multiple cell adhesion molecules are likely to take part in encounters between early HSCs and osteoblasts. Furthermore, final affinity and avidity would extend beyond the identification of a candidate molecule, inasmuch as many adhesion molecules exist in multiple isoforms and glycosylation states and can exhibit different conformations.

Osteopontin, a highly acidic glycoprotein secreted by osteoblasts, is involved in events within the hematopoietic niche [86, 110]. Osteopontin has multiple functions, including involvement in cell adhesions, angiogenesis, apoptosis, inflammatory responses, and tumor metastasis; until recently osteopontin was not known to have a major role in regulating HSC proliferation [86]. Osteoblasts appear to be the sole cells that give rise to osteopontin in the bone marrow microenvironment. This glycoprotein's multiple functions can be ascribed to various post-translational modifications of the binding sites in receptors. It is linked to bone mineralization and resorption through Runx-2 and Notch signaling pathways [110]. Osteopontin's role in hematopoiesis is to inhibit HSC proliferation, possibly to serve as a chemoattractant and an adhesion molecule [86, 110, 111]. In vivo knock-out studies suggest that this glycoprotein contributes to maintenance of HSC quiescence.

Lack of oxygen in the endosteal regions may regulate hematopoiesis there. For example, when oxygen tension was reduced from normal to 1–3%, hematopoietic output was increased 90- to 200-fold [88]. Conceivably hypoxia protects HSCs from the build-up of free oxygen radicals. When pimonidazole was used to identify regions of hypoxia, the highest concentration of HSCs was localized in low-oxygen niches of the bone marrow. HSCs are characterized by high expression levels of the calcium-sensing receptors, N-cadherin, Notch 1, p21, Tert, Bcrp, and low expression levels of p53, p38, and mTOR. Importantly, p21 maintains quiescence, while p53 reduces oxidative stress on HSC populations, perhaps because of its antioxidant functions [46]. In contrast to low-reactive oxygen species (ROS) HSC populations, high-ROS HSCs have increased expression levels of mTOR and phosphorylated p38+ cells. Interestingly, these populations can be recovered by inhibiting p38 with rapamycin or mTOR, which increase the antioxidant activity. Therefore, the unique hypoxic properties of the endosteal niche are likely to contribute to the low-ROS HSCs [46].

Using a slightly different genetic approach, Suzuki et al. [115] established GFP "knock-in" mutant lines in which the GFP cDNA was inserted into the *GATA2* first exon by germline targeting. As expected, HSCs were isolated by monitoring GFP expression from the knock-in mice. Real-time monitoring of *GATA2*-expressing HSCs in living bone marrow showed that individual GFP-positive cells lay in a G0/G1 cell cycle state, in intimate contact with osteoblasts next to the endosteum, at the edge of the bone marrow [115]. Suzuki and co-workers have concluded [115] that the HSC niche is composed of solitary cells and that adult bone marrow HSCs are not clustered in the marrow.

In a recent work by our group [49], osteoblastic membrane proteins were separated on non-denaturing discontinuous gels and blotted with labeled hematopoietic cell lines, resulting in two major adhesive bands, annexin II (Anxa2)

monomers and multimers. Anxa2 was found to be preferentially expressed by osteoblasts at the endosteal surfaces and by endothelial cells in the marrow [49]. Antibody against Anxa2 blocked the adhesion of HSCs to osteoblasts. Parallel results were seen with Anxa2 siRNA knock-down. Overexpression studies demonstrated decreased or enhanced binding. These findings were verified with the aid of primary human HSCs and osteoblasts where the binding of the progenitor cells was linked to Anxa2 [49]. Peptide mapping demonstrated that the majority of the binding activity resides in the N-terminal aspect of the peptide. Most critically, studies of HSC engraftment (i.e., short-term HSCs lodging in the marrow) and multilineage repopulation studies demonstrated a sharp decline in HSC activities when Anxa2 N-terminal peptide fragments or antibodies were used in transplantation of marrow in lethally irradiated animals. Together, these in vitro and in vivo data suggest a novel role for Anxa2 in the marrow microenvironment and niche selection (Fig. 1.1).

1.5 Vascular Niches

Recent work by several groups suggests that in addition to partitioning the vascular and extravascular marrow spaces, bone marrow endothelial cells (BMECs) also regulate HSCs by functioning in a niche capacity. Evidence for this function comes from immunohistochemical studies and the use of simple marker combinations. These are called SLAM family receptors, and include CD150, CD244, and CD48. By means of these, HSCs have been identified as in close physical association with endothelial cells [53, 136]. A BMEC niche for HSCs would be in a position to rapidly mobilize large quantities of stem cells to the periphery so as to respond to systemic challenges. However, whether this is the case and whether there is circulation or communication between the vascular and endosteal or osteoblastic niches has been difficult to establish [40, 57, 58].

Dar and colleagues [20] have shown that CXCR4, expressed on the BMECs, plays an important role in the homing of human CD34+ hematopoietic progenitors to the bone marrow by causing circulating stromal-derived factor-1 (SDF-1 or CXCL12) to translocate to the bone marrow. CXCL12 has been shown to regulate HSCs in both vascular and endosteal niches [92, 94, 96]. By secreting growth factors such as the insulin-like growth factor binding protein-3 (IGFBP-3) BMECs induce HSC proliferation and expansion [14]. This implies that endothelial cells regulate HSC function and contribute to the creation of HSC niches, as they do in the nervous system [53, 136].

BMECs also play a critical role in thrombopoiesis [5], with CXCL12 and fibroblast growth factor-4 (FGF-4) inducing the localization and adhesion of megakaryocytes to the BMECs. Direct contact between megakaryocytes and BMECs promotes survival, maturation, and release of platelets through the VE-cadherin and/or VLA-4/VCAM-1 axis [5], which therefore likely plays a critical role in HSC trafficking [9, 93, 127, 135].

It has been suggested that HSCs in different niches are in different states of activation. According to one model, osteoblasts maintain the quiescent state of HSCs, whereas BMECs cause HSCs to differentiate and proliferate. If this is true, HSCs would be more deeply quiescent in an endosteal than in an endothelial niche. Then, in response to stress (infection, allergy, or bleeding), HSCs would proliferate in the endothelial niche or migrate there, ultimately entering the circulation. The reverse process would then constitute a means to induce quiescence in endosteal sites or niches. As yet this process remains speculative, but is currently subject to intense investigation.

1.6 Other Marrow Niches

Adipocytes: Apart from the well-characterized interactions of osteoblasts and endothelial cells and their associated niches, little is known about the adipocytes that constitute the most prevalent cell type in the marrow microenvironment. There is ample evidence that adipokines play an important role in regulating hematopoietic processes.Adipocytes are always present in long-term cultures, and their

presence is thought to facilitate lymphopoiesis and granulopoiesis. Following lethal irradiation of the host, adipocytes make their appearance after 7 days, as is also true at the beginning of hematopoiesis. Adipocytes secrete soluble factors (IL-6, IL-8, prostaglandin, leptin) that are critical components in hematopoiesis.

Adiponectin may have a role in hematopoiesis. It is produced by differentiated adipocytes, osteoblasts, fibroblasts, and, along with other cell types, occurs in the hematopoietic niche [23]. Activation of adiponectin receptors (AdipoR1, AdipoR2, and T-cadherin) leads to insulin sensitivity, glucose uptake, and fatty acid oxidation; this indicates a role in cellular processes that require high energy, for example, proliferation. Cells in the marrow microenvironment express adiponectin, and HSCs express adiponectin (AdipoR1) receptors for which adiponectin is the sole ligand. Adiponectin supports normal HSC function and proliferation [23]. When HSCs enter the G1 phase of the cell cycle, AdipoR1 is upregulated, an indication that adiponectin promotes proliferation. If this signaling pathway is impaired, as in the AdipoR1 knock-down animal, HSCs fail to proliferate or to reconstitute lethally irradiated hosts.

Adiponectin impacts HSC function via p38 MAPK pathways. When the p38 pathway is inhibited, adiponectin-mediated proliferation is abolished. When the p38 pathways are activated, as via IL-3, erythropoietin, G-CSFs and HSC proliferation is enhanced. Thus, adiponectin-mediated activation of p38 is critical to the proliferation events in hematopoiesis [23].

1.7 Reticular Cells

Reticular cells are among the least understood marrow cells. Under normal steady-state conditions, they express alkaline phosphatase, but not α-SMA. Studies suggest that these large cells represent specialized pericytes of venous sinusoids in the marrow. Adventitial reticular cells of venous sinusoids accumulate lipids and convert to adipocytes [12]. These cells may also be a major source of the regulatory protein CXCL12. Using a 'knock-in' approach Sugiyama et al. showed that many HSCs are in contact with cells that express high amounts of CXCL12. The authors term these CXCL12-abundant reticular (CAR) cells. Because CAR cells surround sinusoidal endothelial cells or are located near the endosteum, they appear to be a key component of both vascular and endosteal niches in adult bone marrow.

1.8 HSC Homing to the Marrow and Occupancy

It is well documented that HSCs 'home' to bone during fetal life and during transplantation [98, 123]. Of the numerous chemokines and molecules that regulate stem cell maturation, trafficking, and homing [6], CXCL12 (SDF-1α and SDF-1β) belong to the C-X-C chemokine family and were originally isolated from a murine BMSC line [80]. SDF-1α and SDF-1β are derived by alternative splicing of the CXCL12 gene and have similar biological activities. CXCL12 is widely expressed in many tissues during development, its expression often juxtaposed with that of its receptor (CXCR4) [75]. A different CXCL12 receptor, CXCR7/RDC1, has been cloned and reported, but little is known about its role with respect to HSCs [7, 69]. CXCL12 and CXCR4 appear essential for bone marrow formation [3, 54]. Hematopoiesis is normal in the fetal liver of CXCL12 or CXCR4 knock-out animals, but hematopoietic cells do not become engrafted [3, 79, 94]. CXCL12 is a powerful chemoattractant for mature and early hematopoietic cells that express the CD34 antigen [43]. CXCL12 also promotes survival of clonogenic progenitors [64, 65]. Overexpression of CXCR4 by human hematopoietic progenitor enhances and anti-CXCR4 antibodies inhibit marrow engraftment in nude mice [94]. Thus, CXCL12 is critically important in establishing hematopoiesis in marrow niches.

Molecules responsible for the regulation of cell trafficking, e.g., CXCL12 and its receptor CXCR4, have dynamic and complementary expression patterns during organogenesis [75]. Osteoblasts and marrow endothelial cells express CXCL12, which then leads to drive

hematopoietic progenitor cells into the marrow [35, 94, 130]. In the bone marrow, CXCL12 is constitutively produced by osteoblasts, fibroblasts, endothelial cells, and possibly CAR cells [48, 95, 113]. Osteoblast-produced CXCL12 may cause circulating osteoclast precursors to move into bone marrow and to migrate to perivascular stromal sites where they would activate nuclear factor-kappaB ligand (RANKL) to induce differentiation into osteoclasts [138, 139]. Because marrow formation is likely to require coordinated action of osteoblasts and osteoclastic precursors, the absence of osteoblasts producing CXCL12 or of hematopoietic cells expressing CXCR4 could limit osteoclastic and subsequent HSC recruitment into the marrow cavity. Thus, CXCL12 and CXCR4 represent important determinants for bone marrow homing by hematopoietic cells (Fig. 1.1).

1.9 Parasitism of the Niche by Cancers

Many neoplasms tend to metastasize to bone. These include prostate cancer, breast cancer, neuroblastoma, and others that lead to significant morbidity and mortality. However, what the molecular and cellular processes are that cause tumors to metastasize to the marrow remains unclear. Multiple factors are certain to be involved, including the acquisition of the ability to metastasize, chemotactic responses to bone-derived factors, preferential adhesion to bone marrow endothelium, and the interaction of cancer cells with the bone microenvironment.

Metastasis involves cell-to-cell and cell-to-matrix interactions that are mediated by cell surface adhesion molecules. In addition, specific signaling pathways are essential. The regulation of these chemokines, cytokines, proteases, and adhesion molecules in tissue-specific sites may influence the pattern of metastasis. For example, Kang et al. [50] and Minn et al. [76] have shown that different breast cancer cells have genetic markers that metastasize to either bone or lung. The loss of genetic material may increase the potential for metastasis. For example, if KiSS1 is deleted or rearranged in melanomas and breast cancers, the cells cannot downregulate matrix metalloproteinase-9 (MMP-9). This in turn results in increasing the metastatic behavior of the cancer [51]. Receptor/ligand pairs can also help explain the propensity of certain cancers to metastasize to specific tissue locations, as in the case of CXCR4 and its ligand CXCL12 (Fig. 1.1). CXCR4-expression in breast and prostate cancers provides a chemotactic axis for site-specific metastasis with high CXCL12 activity. Local environmental changes are also critical for tissue-specific metastasis. Changes in the environment that include inflammatory responses, matrix remodeling, increased ROS, and local expression of soluble factors are critical for establishing a microenvironment that is receptive to tumor metastasis [52].

In the niche, tumor and stromal cells secrete soluble factors critical to directing the vascularization network to support tumor growth and subsequent metastasis. VEGF-A is responsible for the activation of bone marrow-derived hematopoietic progenitor cells and endothelial progenitor cells that are required for neovascularization. In the initial growth of a primary tumor, the recruitment of VEGF receptor 2^+ ($VEGFR2^+$) endothelial progenitor cells and $VEGFR1^+$ hematopoietic progenitor cells is essential for the development of microvascular networks within the tumor [52].

Early in the establishment of a pre-metastatic niche, $VEGFR1^+$ hematopoietic progenitor cells cluster in the parenchymal tissue before carcinogenic cells reach the tissue. Clustering is aided by the production of VLA-4 and MMP-9. As the progenitor cells cluster, fibronectin is produced and helps create an environment that permits adhesion and engraftment of metastatic cells [40, 41, 52, 99]. VLA-4 and MMP-9 also degrade the basement membrane so that a pre-metastatic niche can be formed. This alteration of the microenvironment leads to the expression of integrins and chemokines (e.g., CXCL12) which will support the engraftment and proliferation of metastatic cancer cells. Following the engraftment of metastatic cells, $VEGFR2^+$ endothelial progenitor cells are recruited to the pre-metastatic niche to further establish a vascular network [40, 41, 52, 99]. Inhibition of $VEGFR1^+$ prevents the establishment of a

pre-metastatic site. Similarly, when VEGFR2$^+$ is inhibited, the micrometastases that form are small, because there is no vascular network. In summary, VEGFR1$^+$ hematopoietic progenitor cells provide the framework for establishing a pre-metastatic niche, whereas VEGFR2$^+$ endothelial progenitor cells support the vascularization of implanted metastatic tumor cells [52].

Primary tumors release soluble factors that induce the mobilization of non-malignant cells into organ systems in order to play a major role in establishing a pre-metastatic niche in which metastasizing cells can lodge. To accomplish this requires the induction of multiple factors including, but not limited to, chemokines, proteases, adhesion molecules, and inflammatory molecules [41]. Pre-metastatic niches are supported by various soluble factors that promote adhesion and invasion of mobile cancer cells into these environments. One interesting study has shown that TNF-α, TGF-β, and VEGF-A secreted by primary tumor cells induce expression of S100A8 and S100A9, molecules that may be responsible for the specific homing patterns observed, such as metastasis to the lung [41]. Hiratsuka et al. [41] have reported that S100A8 and S100A9 increased mobilization and homing of cancer cells to the lungs, as well as priming the pulmonary tissue for metastatic colonization. The chemokine-induced expression of specific S100-chemokines by a primary tumor is an example of a facilitating mechanism to prime a target tissue so that cancer cells can be established [41]. It is not yet known, however, whether S100A8 and S100A9 are expressed solely at pre-metastatic niches or randomly through the lung. Moreover, because S100-chemokines are expressed at higher levels in the lungs than in liver and kidneys, tumor metastasis involves additional factors [41].

Molecules involved in tumor cell metastasis must first be expressed in endothelial cells of the target organ, because the tumor cells must attach first to the endothelial cells of vessels supplying the bone marrow. CXCL12 expression has been confirmed in endothelial cells of human arterioles and arteries from lung, liver, and bone marrow [95]. In addition, endothelial-derived CXCL12 promotes extracellular matrix-dependent tube formation and vascularization during organogenesis [102, 106]. These results suggest that the CXCL12/CXCR4 axis plays a role in angiogenesis and, in addition, that endothelial-derived CXCL12 stimulates tumor cells that express the CXCR4 receptor.

Work by our group and others has defined the role that CXCL12 and its receptors (CXCR4 and CXCR7/RDC1) play in prostate cancer metastasis [17, 39, 82, 113, 114, 117, 131]. In prostate cancer, CXCR4 expression is associated with a corresponding increase in tumor grade [113]. Moreover, CXCL12 signaling through CXCR4 by activating CD164 and $\alpha_v ß_3$ integrins triggers the adhesion of prostate cancer to BMECs [112]. Also, the tissue levels of CXCL12 and the sites of metastatic prostate cancer lesions are positively correlated. This suggests a selective effect (pelvis, tibia, femur, liver, and adrenals) [114]. Most critically, administration of the antibody to CXCR4 in mice significantly reduces the degree of bone metastasis by prostate cancer cells [114].

The same applies to CXCR7/RDC1 (JH Wang, Submitted). Signals generated by SDF-1 binding induce AKT and MAP kinase pathways [128, 131] and inhibit the secretion of intracellular proteins normally associated with glucose metabolism (phosphoglycerate kinase 1 or PGK1). PGK1 is involved in angiostatin generation [129]. Therefore, when metastasizing prostate cancer cells locate in a region rich in CXCL12, PGK1 secretion is inhibited. This generates an 'angiogenic switch' [129]. Other tumors that metastasize to the marrow have given rise to similar events [29, 31, 50, 59, 70, 78, 103]. Therefore, CXCL12/CXCR4 seems to play a central role in bone metastasis (Fig. 1.1).

1.10 Conclusions

In 1889, Stephen Paget proposed a 'seed and soil' metaphor to explain the marked affinity of cancer cells for different tissues, "when a plant goes to seed, its seeds are carried in all directions; but they can only grow if they fall on congenial soil" [87]. There is obvious similarity between Paget's proposal and the current concept that most cancers likely originate from alterations in somatic

stem cells. This concept is attractive because it explains why many cancers resist cytostatic agents that target rapidly dividing cells, but fail to cure because cancer stem cells, like other stem cells, progress slowly through the cell cycle [15, 60, 100]. The cancer stem cell hypothesis also explains why metastases are relatively restricted inasmuch as it requires that the rather rare stem cells establish metastasis. Germane to the current concept is the notion that if cancers are to metastasize, they need to engage specific locales or 'niches' in remote locations.

Most cancers contain a small number of functionally distinct cancer stem cells. It is an open question, however, what attributes are needed for a cancer stem cell to establish a niche. Stromal cells produce CXCL12 which regulates homing to the bone marrow by HSCs that express CXCL12 receptors (Fig. 1.1). Conceivably the CXCL12 pathway to marrow localization applies more widely (Fig. 1.1).

One common feature of niche or osteoblast-supported hematopoiesis is the requirement for cell-to-cell contact. Indeed HSC survival is strongly dependent on adhesive interactions with osteoblasts in vitro. HSC survival, HSC progression through the cell-cycle, leukemogenesis, and HSC homing to the bone marrow are all linked to adhesive interactions between stem and stromal niche cells (Fig. 1.1). In cancer, as in hematopoiesis, the HSC niche is utilized for localization, growth and survival in bone (Fig. 1.1). Our understanding of tumor–HSC niche interactions is still in its infancy. As in hematopoiesis, so in cancer CXCL12 produced by the niche participates in the regulation of prostate cancer metastasis and proliferation [16, 17, 19, 50, 68, 112–114, 121, 131]. It therefore seems that osteotropism is a result of cancer cells parasitizing the HSC niche. This concept needs more study.

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2.

Genetics of Osteosarcoma

Marc F. Hansen

Primary malignant tumors of bone are rare and constitute one of the more uncommon types of neoplasms. Only about 1,500 new bone sarcomas are reported in the United States each year. Yet, because of the effects of radical surgery and chemotherapy, the very existence of these tumors leads to a significant reduction in the quality of life in children and adolescents. Notwithstanding their rarity, primary tumors are important for understanding cancer and its treatment.

Osteosarcoma is the most common primary tumor of bone and accounts for approximately 19% of all malignant tumors in bone and 40–60% of all primary malignant tumors of bone [59, 128, 186, 236, 297]. It is the most common solid tumor in teenagers and the third most common malignancy in children, accounting for 7% of all adolescent cancers [321]. Twenty years ago, the advent of a multidisciplinary approach that combined multi-agent chemotherapy and limb-sparing surgery greatly improved the survival rate of patients with osteosarcoma. Sadly, since then the 5-year survival has plateaued at approximately 70% and outcome has not improved significantly; indeed, long-term complications of osteosarcoma survivors treated with intensive chemotherapy have increased [90]. Furthermore, the prognosis for patients with metastatic disease or those with local relapse is much worse; if patients develop extrapulmonary metastatic disease, they almost never survive [12, 128].

2.1 Histopathology of Osteosarcoma

The defining characteristic of osteosarcoma is the production of osteoid [297]. Beyond this, osteosarcoma can be divided into several subtypes based on histopathological and clinical features. Most broadly, the tumors can be divided into those that arise within the bone (intramedullary) and those that arise on the surface of the bone [199]. Most intramedullary osteosarcomas are highly malignant and most frequently occur during adolescence [297]. In contrast, most osteosarcomas that occur on the surface of the bone tend to be less aggressive and contain cells that are either well or moderately well differentiated.

Intramedullary osteosarcoma tumors are typically localized to the metaphyseal portion of the long bones, with the majority of tumors occurring in the distal femur and proximal tibia. These tumors can have predominant elements of osteoblastic, chondroblastic, or fibroblastic differentiation (Fig. 2.1). Other histopathological features include a small round cell variation [10, 11, 202] and a variation with giant osteoclast-like cells [19, 201, 253]. The molecular and/or genetic bases of these histologic variations have yet to be systematically explored.

Osteosarcoma is characterized by osteoblast-like tumor cells that produce a disorganized field

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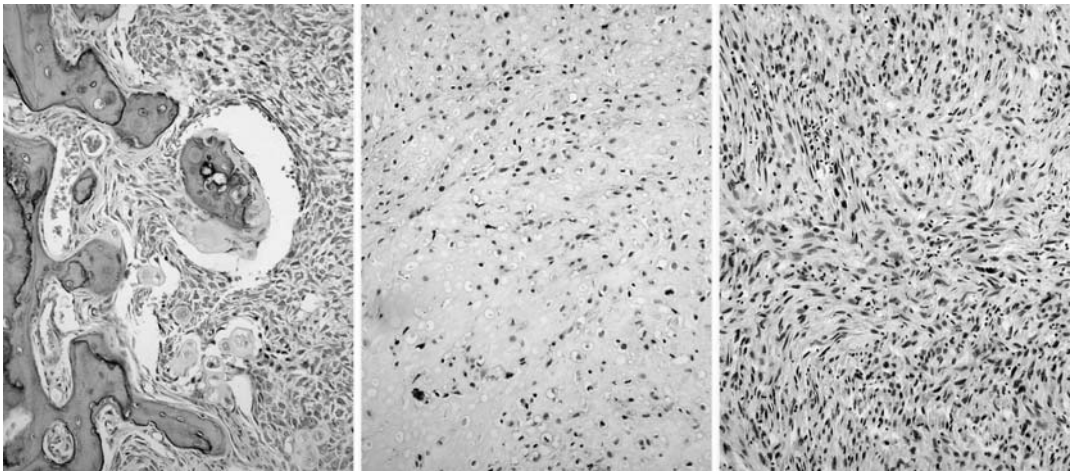

Figure 2.1. Histopathology of conventional intramedullary osteosarcoma. Shown left to right are examples of osteoblastic, chondroblastic, and fibroblastic subtypes of conventional intramedullary osteosarcoma. In each example, obviously anaplastic cells and osteoid production can be detected. In the osteoblastic example, reactive bone is found retained within the neoplasm and serves as a scaffold upon which osteoid is deposited by the neoplastic cells. In the chondroblastic subtype, osteoid production by neoplastic cells requires careful search to be detected in the cartilaginous material. In the fibroblastic subtype, there is an admixture of pleomorphic spindled cells and enlarged polygonal cells and only focal presence of osteoid.

of calcified tissue, including osteoid and bone. Osteosarcoma tumors can be highly cellular, with little osteoid production, or sparsely cellular, with abundant calcified osteoid matrix. Unusual or undifferentiated tumor cells occur frequently in osteosarcoma tumors, as are foci of neoplastic cartilage or fibrous tissue. This can result in misdiagnosis of chondrosarcoma or fibrosarcoma in poorly sampled pathological specimens.

Osteoid production is associated with well-vascularized tumors, whereas malignant cartilage is more commonly associated with poorly vascularized tumors. This may be one reason that chondroblastic differentiation is associated with a slightly worse response to chemotherapy than other types of intramedullary osteosarcomas, owing to poor delivery of the drug. Predominantly osteoblastic tumors are typically sparsely cellular, with unusual mineralized matrix, and are in juxtaposition to native trabecular and cortical bone. Sheets of tumor cells are pushed against malignant bone with no osteoblasts lining the surface.

Although cell types vary, osteosarcomas have in common cytological characteristics such as pleomorphism, hyperchromatism, and abundant atypical mitoses. Epithelial-like cells have been found in some osteosarcomas. This finding suggests that some osteosarcomas arise from primitive pluripotent mesenchymal stem cells [151]. Other osteosarcomas appear to arise from mesenchymal stem cells with rhabdomyosarcomatous-like or lipomatous-like features [154, 177, 196, 243], or from more committed osteoprogenitor lineage cells [30].

Unfortunately, the histopathological classification has little or no prognostic significance. Osteosarcomas also can be divided into sclerotic and lytic subgroups, but this too has no value in clinical prognosis. At present, more than 80% of patients with appendicular osteosarcoma with no distant metastases will become long-term survivors [152].

2.2 Unconventional Osteosarcoma Subtypes

One unusual subtype of intramedullary osteosarcoma is telangiectatic osteosarcoma [185, 200] (Fig. 2.2). The tumor is almost completely lytic in appearance, resembling an aneurysmal bone cyst with large hemorrhagic cystic cavities that contain blood clots, tumor fragments, and tissue debris. Curiously, these tumors appear to arise in the metaphysis and then to extend into the diaphysis. Histologically,

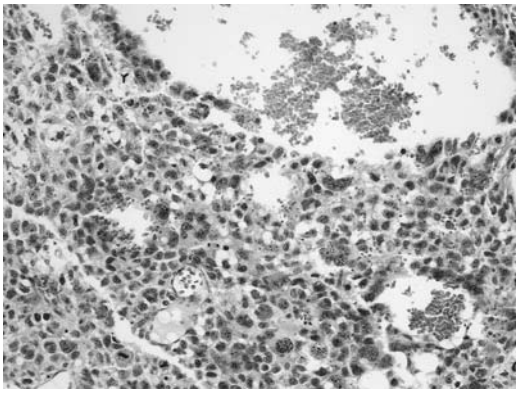

Figure 2.2. Histopathology of telangiectatic osteosarcoma. Note the characteristic cystic vasculature spaces at the top and right of the example. Osteoid production is minimal. These tumors can be confused with aneurysmal bone cysts.

the hemorrhagic cysts contain tumor cells and giant cells that line the septa of the cysts. Osteoid is produced by the tumor cells within these cysts. Response of these tumors to chemotherapy appears to be similar to that of other intramedullary osteosarcomas [13, 317].

Surface osteosarcomas arise and are confined to the surface of the bone and do not involve the medullary canal. They are divided into three categories: periosteal, parosteal, and high-grade surface osteosarcomas [132, 241, 254]. Periosteal osteosarcoma typically occurs as a diaphyseal lesion on the tibia or femur and can be mistaken for periosteal chondrosarcoma [94, 227, 245, 246]. Histologically, periosteal chondrosarcoma is composed of lobules of atypical proliferation, with the center of the tumor displaying mineralization, whereas the peripheral portions of the tumor tend to be composed of proliferative spindle-shaped cells. Whether chemotherapy affects the long-term outcome of periosteal osteosarcoma is controversial [85, 132].

Parosteal osteosarcoma is a slow-growing, relatively indolent tumor [166, 218, 262,299] that is densely mineralized and envelops the shaft of the bone. It is characterized by low-grade fibroblast-like spindle cells with minimal cellular atypia that line the long axis of the bone with embedded sheets of fibrous stroma. The osteoid that lines the tumor merges with the underlying fibrous tissue. Parosteal osteosarcoma rarely metastasizes, with recurrence locally the major risk. Occasionally, indolent tumors become anaplastic. The resulting condition is designated dedifferentiated parosteal osteosarcoma [1, 262, 280, 322]. The dedifferentiated component is characterized by a pleomorphic spindle cell phenotype. Although surgical resection appears sufficient for the more indolent tumor, adjuvant chemotherapy is recommended for the dedifferentiated form of the disease [262].

High-grade surface osteosarcomas are rare variants of the surface osteosarcoma. The tumors appear similar on histological examination to conventional intramedullary osteosarcomas, except that they are confined to the surface of the bone. Osteoid and bone production also are similar to intramedullary tumors. Outcome is generally similar to intramedullary osteosarcoma [113, 219].

Extraskeletal osteosarcomas provide an interesting insight into the disease. They arise within the muscle or soft tissues, usually of the thigh and buttock regions, and do not involve bone [2, 14, 35, 53, 157, 164] (Fig. 2.3). Mean age of onset is later than the bony osteosarcomas. Histologically, the tumors present with any of the differentiation patterns of intramedullary osteosarcoma: chondroblastic, fibroblastic, osteoblastic, small cell, giant cell-rich tumors, or even telangiectatic phenotypes [53]. It is tempting to think these tumors arise from mesenchymal stem cells located within the soft tissues and undergo osteoprogenitor differentiation as part of their tumorigenic process.

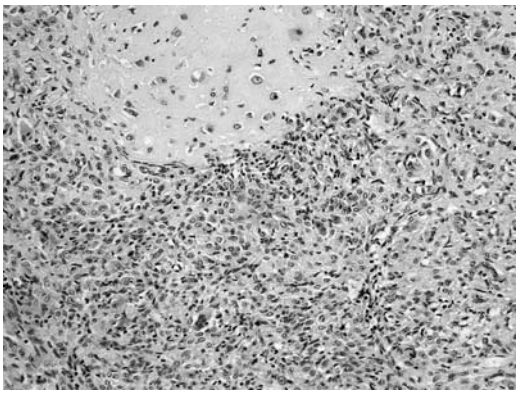

Figure 2.3. Histopathology of extraskeletal osteosarcoma. Note the chaotic mixture of cell types that compose the tumor.

2.3 Head and Neck Osteosarcoma

About 6–13% of osteosarcomas occur in the head and neck, with the most common site being the mandible, followed by the maxilla and the other bones of the skull [37, 60, 123, 178]. Craniofacial osteosarcoma can be either primary, i.e., arise in the absence of known predisposing factors, or secondary, i.e., arising in response to and arising within radiation fields (as in radiation-treated bilateral retinoblastoma patients) or in response to other disease conditions, such as Paget's disease [183]. Secondary osteosarcomas of the head and neck are aggressive lesions that are clinically similar to osteosarcomas of the long bones.

Appendicular osteosarcomas occur between the ages of 10 and 18, coinciding with the major post-pubescent growth spurt [230]. Primary craniofacial osteosarcomas have a median onset in the fourth decade of life [18, 24, 60, 123, 178, 216, 269, 284, 304]. Appendicular and secondary craniofacial osteosarcomas metastasize widely within a year of the initial diagnosis. It is the distant metastases that are the most common cause of death. In contrast, primary craniofacial osteosarcomas do not metastasize aggressively and spread more slowly, with the mean interval between initial treatment and discovery of a metastatic lesion some 20+ months [24]. Local recurrence is the major complication and the leading cause of death in primary craniofacial osteosarcomas. Appendicular osteosarcoma and secondary craniofacial osteosarcoma are both characterized by pronounced cellular atypia. Histologically, craniofacial osteosarcomas are most frequently chondroblastic in appearance [60, 127], show little cellular atypia, and are frequently confused with benign or reactive bony lesions (Fig. 2.4). Neither pathologic staging of primary craniofacial osteosarcomas nor extension of the osteosarcoma into the surrounding soft tissues correlates well with survival. In many cases, only the completeness of the surgical resection as determined by margin status has correlated well with outcome, whereas incomplete resection correlates with local relapse and poor survival. In osteosarcomas of the head and neck, tumors of the mandible excepted, it is difficult to achieve complete surgical resection. Osteosarcoma of the mandible, therefore, has a better prognosis than other types of craniofacial osteosarcoma. However, even in patients with mandibular osteosarcoma, complete surgical resection is achieved in only one-third of the cases [15]. The reason for the low rate of complete resection is extension of the tumor into adjacent structures, which occurs in 50% of patients with mandibular osteosarcoma.

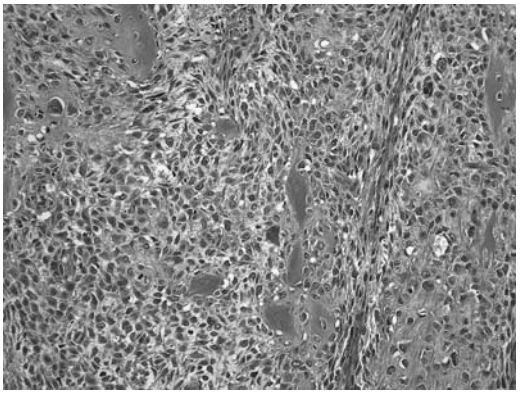

Figure 2.4. Histopathology of primary osteosarcoma of the mandible.

One of the possible reasons for the difference in phenotype between primary craniofacial osteosarcomas and appendicular osteosarcomas is that the bones of the head and neck undergo a different program of development from those of the long bones of the skeleton [36, 39, 55]. In the precursors of the craniofacial bones (the calvaria of the skull, the maxilla, and the mandible), neural crest-derived cells differentiate into osteoblasts in a process known as intramembranous ossification. In the appendicular portions of the skeleton, mesenchymal cells differentiate into bone through a process called endochondral ossification. This difference in origin may be reflected in the distinct clinical and biological behavior of the two tumor types.

2.4 Osteosarcoma and Bilateral Retinoblastoma

Predisposition to osteosarcoma has been associated with several inherited syndromes.

Kitchin and Ellsworth [142] had observed that patients with bilateral retinoblastoma were at an increased risk for secondary tumors, notably osteosarcoma, whether or not the patient had been treated with radiation for the first tumor. They concluded that the increased risk was due to the pleiotropic effect of the susceptibility for retinoblastoma. This hypothesis was strengthened by the discovery that osteosarcoma tumors from patients with bilateral retinoblastoma lost constitutional heterozygosity (LoH) in the same region of chromosome 13 as in retinoblastoma tumors [50, 96, 256].

Cloning the gene for retinoblastoma susceptibility (RB1) demonstrated that the association between retinoblastoma and osteosarcoma was due to mutations in a common gene called RB1 [65, 66, 121, 160], consistent with its role as a tumor suppressor [7, 257, 307, 316, 327]. Furthermore, reintroduction of the RB1 gene into osteosarcoma tumor cells resulted in reduced tumorigenicity, both in vivo and in vitro [114].

2.5 Osteosarcoma and Li–Fraumeni Syndrome

The second association between osteosarcoma and an inherited predisposition was detected in the cancer syndrome first described by Li and Fraumeni [162]. These investigators and others [102, 163, 234] identified osteosarcoma as one of the more common tumors associated with rhabdomyosarcoma, breast cancers, and other neoplasms. The link between these disparate tumors was first suggested by the discovery of mutations in the TP53 gene in sporadic osteosarcoma tumors [181]. This was followed by the discovery of inherited mutations in the TP53 gene in several familial Li–Fraumeni syndromes [172]. As with RB1, TP53 is frequently mutated in sporadic osteosarcomas [189, 190, 291] and insertion of TP53 into osteosarcoma tumor cells has led to a loss of tumorigenicity in vivo and in vitro [48].

Li–Fraumeni syndrome, a heterogeneous disease, is associated with inherited mutations in the CHK2 gene in some families [159]. Activated CHK2 stabilizes TP53, as well as acting on other genes in the TP53 pathway. Inherited mutations in the CHK2 gene have been identified in sporadic osteosarcomas and in osteosarcomas in families with Li–Fraumeni syndrome [191].

2.6 Osteosarcoma and Rothmund–Thomson Syndrome

Osteosarcoma is also associated with a rare autosomal recessive syndrome termed Rothmund–Thomson syndrome [249, 290], characterized by progressive poikilodermatous skin changes, juvenile cataracts, and skeletal abnormalities [305]. Individuals with this syndrome have an increased incidence of malignancies, including osteosarcoma. The predisposing mutation involves mutations in a helicase gene RECQL4 [141] and other mutations in RECQL4 [16, 167]. Also, osteosarcomas in Rothmund–Thomson patients were found associated with truncation of the RECQL4 gene [311]. Curiously, in contrast with the osteosarcomas associated with RB1 and TP53, sporadic osteosarcomas were not associated with mutations in RECQL4 [213].

An increased risk of osteosarcoma has also been associated with Werner's syndrome, caused by mutations in the related helicase, WRN/RECQL2 gene [80, 198]. Osteosarcoma may therefore be sensitive to changes in DNA repair that result in chromosomal instability.

2.7 Osteosarcoma and Paget's Disease of Bone

Osteosarcoma also has been associated with Paget's disease. Paget's disease is the second most common metabolic bone disease that affects up to 4% of the U.S. population by age 60 [63, 265, 266]. Rapid bone turnover in this condition alters the strength and shape of the newly formed bone [63, 226, 242, 265, 266]. The familial form of the disease is inherited in an autosomal dominant fashion with variable penetrance [261, 267]. Predisposition to familial Paget's disease has been linked to a number of loci [43], with osteosarcoma associated in 84% of cases [92, 118, 193, 235, 270]. Pagetic sarcoma

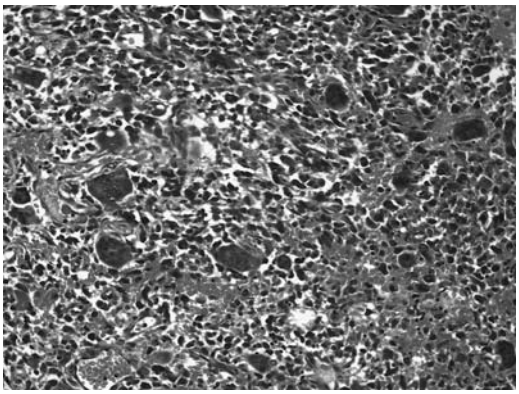

Figure 2.5. Histopathology of pagetic osteosarcoma. Note the presence of large osteoclast-like giant cells within the tumor.

occurs in 0.7–5% of patients with Paget's disease [64, 82, 91, 93, 319]. Osteosarcomas related to Paget's disease account for approximately 3% of all osteosarcomas [298], but account for 20% of all osteosarcomas in patients over 40 years of age [319] and for 50% of osteosarcomas in patients over 60 years of age [117].

Most osteosarcomas that develop in the Pagetic bone are conventional high-grade intramedullary tumors characterized by a highly pleiomorphic, often spindle-cell sarcoma [82] (Fig. 2.5). The tumors are marked by the presence of many osteoclastic giant cells and atypical osteoblasts that seem responsible for the high rate of bone remodeling typical of Paget's disease.

The molecular basis for the increased risk for osteosarcoma in Paget's disease is unclear [97]. Analysis of LoH patterns identified a putative tumor suppressor locus in the same region of chromosome 18q that has been implicated as predisposing to some forms of familial Paget's disease [126, 184, 207], but no common mutations have been identified.

2.8 Genetics of Osteosarcoma

Osteosarcoma, despite its relative rarity, has played a significant role in the discovery of tumor suppressor genes such as *RB1* [50, 65, 66, 96, 160] and *TP53* [48, 189], as well as in the discovery of proto-oncogenes such as *FOS* [41, 42, 76, 84, 187, 250, 303, 313, 314] and *MDM2* [190, 222]. Indeed, many more cancer genes have been identified in leukemias, lymphomas, and sarcomas than in any other type of cancer, even though they account for only 10% of human cancers [67].

2.9 RB1 and Osteosarcoma

RB1 was the first human tumor suppressor gene to be cloned, but its mechanistic role in tumorigenesis remains incompletely understood. RB1 plays a role in many cell processes, including cell cycle regulation [99, 281], DNA damage response and repair [69, 144, 145, 310], DNA replication [210], apoptosis [32, 99, 107], and differentiation [38, 45, 148, 168].

Mutations in RB1 in osteosarcoma were some of the earliest mutations detected in RB1 [65, 66, 160]. Subsequent analysis has shown that inactivation of RB1 is the most common mutational event in osteosarcoma [45, 307, 316, 327].

RB1's role in regulating cell cycle progression may be through its repression of gene expression mediated by E2F1 and other members of the E2F family of transcription factors [54]. However, RB1 also regulates gene expression by recruiting chromatin remodeling complexes to promoter regions that mediate chromatin condensation and inhibit transcription [22, 23, 98, 100].

RB1 is regulated by a group of cyclin-dependent kinases (CDKs) in response to mitogenic stimulation during cell cycle progression, allowing the cell to pass through the G1/S boundary [46]. RB1 phosphorylation leads to disruption of the RB1/E2F1 association and depression of a variety of E2F1-regulated genes. This, in turn, leads to a proliferative response. CDKs are regulated by a group of CDK inhibitors, which prevent CDKs from phosphorylating RB1. Mutations in the CDKI proteins [188, 212], as well as amplification of CDK genes [68, 130, 137, 238, 315, 328], have been found in some osteosarcomas; this suggests alternative mechanisms to inactivate the RB1 pathway.

A Pne alternative regulatory mechanism has been identified. During apoptosis, RB1 is degraded by caspases in response to TNF-alpha-

mediated apoptotic signals [282, 283]. This leads to derepression of the E2F1-regulated gene APAF1 [87, 195]. APAF1 is a key component of the mitochondria-dependent apoptotic machinery [27, 312, 337]. However, thus far no mutations in APAF1 have been identified in human osteosarcoma.

2.10 TP53 and Osteosarcoma

TP53 is one of the most commonly mutated genes in human cancer [276, 292, 306]. Mutations leading to inactivation of TP53 are common in osteosarcoma tumorigenesis [120, 134, 181, 189, 190, 224, 229, 231, 237, 257, 308]. As described previously, germline mutations in TP53 can predispose to osteosarcoma.

TP53 plays a crucial role in a number of pathways related to cellular stress, DNA repair, and apoptosis [276]. TP53 induces cell cycle arrest, senescence, differentiation, and apoptosis depending on the genetic environment of the cell. In response to genotoxic damage, TP53 can contribute to DNA repair. However, most often induction of TP53 by genotoxic damage leads to irreversible activation of apoptosis. TP53 function can be lost through mutation of the TP53 gene, or through mutations of genes within the TP53 signaling pathway [101]. TP53 is regulated by MDM2, a protein that blocks the activity of the TP53 protein by directing it to the ubiquitin-mediated degradation pathway [25, 122, 292]. MDM2 is negatively regulated by p14ARF [333], whereas CHK2-mediated phosphorylation of TP53 prevents MDM2 inactivation of TP53 [233]. Overexpression of MDM2, which results in functional loss of TP53 activity, occurs in osteosarcoma [134, 137, 190, 206, 229, 231, 238, 315, 328] as do mutations in the p14ARF and CHK2 genes that lead to functional inactivation of these genes [17, 170, 191].

2.11 Wnt Signaling Pathway

Signaling through the canonical Wnt pathway is critical for the differentiation of progenitor cells into osteoblasts [73, 74]. During osteogenesis, stimulation by bone morphogenic protein 2, a bone differentiation factor, is sustained by Wnt signaling. When Wnt signaling is inhibited, mesenchymal stem cells enter the cell cycle and osteogenesis is breached. Dickkopf 1 (DKK1) disrupts the Wnt signaling cascade [211], resulting in the inhibition of osteogenesis [83].

Serum levels of DKK1 are significantly elevated in pediatric osteosarcoma patients [158], with DKK1 expression at a maximum in the osteosarcoma cells located at the periphery of the tumor. When human mesenchymal cells are cultured in conditioned media from osteosarcoma tumor cells, osteogenesis is reduced in the same fashion as when DKK1 is added. Immunodepletion of DKK1 or addition of an inhibitor blocks the inhibitory effect on osteogenesis [158].

The level of expression of LRP5, a co-receptor in the Wnt signaling pathway, has been found to correlate positively and significantly with a rise in tumor metastasis. Patients whose tumors were positive for LRP5 tend to have a lower level of event-free survival [109]. Expression of Dickkopf 3 (DKK3), a dominant-negative mutant of LRP5, reduced invasion and motility in an osteosarcoma tumor cell line by modulating the Wnt-beta-catenin pathway [110]. Specifically DKK3 upregulated E-cadherin and downregulated Slug and Twist, transcription factors associated with regulation of metastasis. DKK3 expression also led to reduced expression of matrix metalloproteinases MMP2 and MMP14, as well as of Met and hepatocyte growth factor (HGF), enzymes that are involved in invasion and cell motility [86].

Wnt signaling therefore may play an important role in osteosarcoma tumorigenesis by inhibiting repair of the surrounding bone and by increasing the motility and invasiveness of the tumor cells.

2.12 Ezrin and Metastasis

Ezrin is a gene associated with motility, invasion, and adherence. Together with radixin and moesin, it is a component of the ERM proteins, which act as links between the plasma membrane and the actin cytoskeleton [116]. The ERM

proteins are involved in cell adhesion, migration, and the organization of cell surface structures. The role of Ezrin in osteosarcoma tumorigenesis was discovered by way of a microarray analysis of a mouse model of osteosarcoma [135]. Subsequent analyses have shown that Ezrin is overexpressed in aggressive mouse and canine tumors, as well as in metastatic human osteosarcoma tumors [135, 136, 140, 161, 228, 251].

Ezrin expression provides an early survival advantage for metastatic osteosarcoma tumor cells that reach the lungs in that AKT and MAPK phosphorylation and activity were reduced when Ezrin protein was suppressed [136]. Khanna and colleagues [136] also found that Ezrin-mediated early metastatic survival was partially dependent on activation of MAPK, but not of AKT.

Another member of the ERM protein family, Merlin, the product of the NF2 gene, is linked to highly metastatic osteosarcomas in mice [182]. This is surprising, as mutations in the NF2 gene in humans do not show increased predisposition to osteosarcoma. Moreover, analysis of NF2 in human osteosarcoma has not detected any mutations [274]. Possibly, another member of the ERM protein family compensates for loss of Merlin function in human osteoblasts.

2.13 FAS and FASL Signaling

The FAS receptor and its ligand (FASL) belong to the tumor necrosis factor death receptor superfamily and participate in regulating tumorigenesis in several types of primary malignancies and metastases [309]. Low expression of FAS in different tumors, including osteosarcoma, correlates with poor prognosis. Osteosarcoma lung metastases express low levels of FAS, whereas the primary tumors from the same patients often express high FAS levels [77, 149, 153]. In mouse models of osteosarcoma, FAS expression and metastatic potential were consistently found to vary inversely [78, 326]. One explanation is that FASL is constitutively expressed in lung tissue and that FAS-positive osteosarcoma tumor cells that enter the lungs bind to the FASL and induce apoptosis [78, 149]. This explanation is consistent with the earlier observation that cyclophosphamide and its derivative ifosfamide induce expression of FASL in osteosarcoma cells [52]. Induction of FASL mediates apoptosis in osteosarcoma tumor cells via an autocrine–paracrine loop by cross-linking with cell surface FAS. Duan et al. [51,52] also showed that IL-12 enhanced the sensitivity of osteosarcoma cells to cyclophosphamide by upregulating FAS. This is consistent with FAS/FASL regulation in osteosarcoma, inasmuch as cells with high FAS expression are likely to be more sensitive to agents that upregulate FASL.

Chemotherapy agents that upregulate FAS would be expected to inhibit lung metastases. Gemcitabine, a pyrimidine antimetabolite and an analog of cytosine arabinoside, caused growth inhibition and cell death in human osteosarcoma tumor cell lines [124]. When mice were treated with an aerosol form of gemcitabine, FAS expression increased and the tumor regressed [5, 78, 124, 150].

2.14 erbB2/HER2 and Its Role in Osteosarcoma

The erbB family of type I protein receptor tyrosine kinases may be one group of genes which, when their mechanism of action is better understood, may lead to the identification of new targets for osteosarcoma therapy. This erbB family consists of erbB1 (also known as the epidermal growth factor receptor EGFR), erbB2 (also known as HER2 or neu), erbB3 (also known as HER3, and erbB4 (also known as HER4) [26, 106, 111, 119, 221]. These cell surface receptors form homodimers and heterodimers [40, 330, 331] to create functional growth factor receptors that trigger more rapid growth in malignant cells [208, 209] and promote cell survival [71].

HER2 is the best known member of the family [111, 209, 247, 248, 268]. It has no known ligands [143], but promotes signaling when combined as a heterodimer with any other family members that have ligands [220, 221, 278, 279]. Other erbB family members will preferentially partner with HER2 when co-expressed [81, 295]. Immunohistochemical examination of HER2 in

breast cancer cells has revealed strong antigen staining along the edges of tumor cells. This is consistent with membrane staining [268]. Overexpression of HER2 is correlated with genomic amplification to the point where identification of *HER2* amplification by fluorescent in situ hybridization (FISH) has been approved by the US Food and Drug Administration as a procedure to identify patients at high risk for recurrence and death due to node-negative invasive breast cancer [129, 332].

HER2 expression and gene amplification in osteosarcoma have been examined in many published reports [3, 4, 6, 58, 79, 115, 139, 171, 223, 271, 289, 294, 300, 320, 334]. The results of these studies appear to be contradictory: several studies report that HER2 plays a prognostic role, whereas other reports show no significance. The difference may be due to the definition of HER2 overexpression. In breast cancer, HER2 cytoplasmic immunostaining is considered to be an artifact [288, 289] and only complete membrane staining is considered to be clinically relevant [20]. Moreover, overexpression must be accompanied by genomic amplification; this has not been routinely observed in osteosarcoma [171], except in a single study that utilized FISH analysis [334]. In general, when HER2 expression in osteosarcoma was examined by immunohistochemistry, the pattern of staining was faint and diffuse; this suggests localization in the cytoplasm rather than in the plasma membrane [115]. Therefore, if and how HER2 expression affects osteosarcoma biology if the receptor is not expressed on the cell surface is unresolved.

2.15 RECQL4 and Genomic Stability

RECQL helicases represent a highly conserved protein family that is needed to maintain genome integrity [95, 108, 205, 301]. Three of the RECQL family members predispose to cancer predisposition syndromes: Bloom's Syndrome, Werner's syndrome, and Rothmund–Thomson syndrome. All three syndromes share a common phenotype of genomic instability [108, 301]. An important function of the RECQL helicases appears to be the unwinding of intermediates of recombination, thereby preventing uncontrolled recombination [205].

Loss of function of the RECQL family of helicases gives rise to an increase in the levels of recombination. This in turn results in chromosomal aberrations that include LOH, a common chromosomal change associated with malignancies [108, 205]. RECQL4 may play a role in initiating DNA replication and in sister-chromatid cohesion [155, 176]. In normal human fibroblasts, RECQL4 is predominantly localized in the cytoplasm; relocation from nucleus to nucleolus or other nuclear foci occurs in response to UV or oxidative cell stress [232, 318, 325]. RECQL4 also associates with RAD51; this suggests that RECQL4 has a role in repairing double-strand breaks of DNA by homologous recombination [232].

One difference between RECQL4 and other mutated genes that predispose to osteosarcoma (RB1, TP53) is that no somatic mutations of RECQL4 have been identified in sporadic cases of osteosarcoma [213]. This may reflect the fact that mutations in RECQL4 would only have an indirect effect on tumorigenesis, whereas RB1 and TP53 have more direct effects.

2.16 Role of Chromosomal Instability and Telomere Maintenance in Osteosarcoma

One of the striking features of osteosarcoma is the high frequency of genomic amplification, rearrangement, deletion, and loss of heterozygosity across the genome [8, 112, 156, 174, 197, 258, 259, 273, 277, 286, 324, 329, 336]. This chromosomal instability is rare in childhood tumors.

Chromosomal instability is common in cancer cells. Mechanisms that lead to numerical and structural chromosomal instability in cancer cells include defects in chromosomal segregation, defects in cellular checkpoints that guard against reproduction of abnormal cells, defects in telomere stability, and defects in the DNA damage response. A long-standing debate in cancer genetics is whether genomic instability is an

early or late event in tumorigenesis [169, 179, 180, 214, 263, 264, 293]. Chromosomal instability has been studied primarily in epithelial tumors, notably colorectal carcinoma. Approximately 15% of colorectal cancers show a form of genetic instability that is characterized by mismatch repair deficiency. The remaining 85% of colorectal cancers, and an even larger proportion of other solid tumor types, show an abnormal chromosomal content that reflects chromosomal instability [240]. Unlike microsatellite instability, which is caused by genes in the DNA mismatch repair pathways, chromosomal instability is due to errors in chromosomal segregation, telomere stability, and in the repair of damage to double-stranded DNA [33, 75, 146, 147, 239, 302].

Alterations in over 100 genes have been shown to give rise to chromosomal instability in *Saccharomyces cerevisiae* [146]. Many of these have one or more homologs in humans. These include those involved in cell cycle regulation, chromosome condensation, sister-chromatid cohesion, spindle assembly, kinetochore structure and function, microtubule formation and dynamics, as well as cell cycle checkpoints.

Alterations in telomeres have been associated with increased genomic instability [44, 180]. Terminal deletions induced by telomere shortening in the absence of telomerase may be initiated by end-to-end chromosome fusion and breakage or by exonucleolytic end resection. In telomerase-deficient mice, end-to-end chromosome fusion is the most prominent chromosomal abnormality [21], with fusions a primary consequence of telomere shortening [104]. In human tumors with telomere dysfunction, deletions in the terminal regions of chromosomes precede an increase in global instability [44, 57, 72].

Decreased telomerase activity leads to chromosomal end lesions, which promote either genomic instability and carcinogenesis or apoptotic cell death [34]. Telomerase may therefore have a dual role in promoting tumorigenesis and protecting the cell from genomic instability [31, 47, 88, 89]. Studies using a model for Li–Fraumeni syndrome have suggested that telomere shortening is the primary driving force for the genomic instability characteristic of Li–Fraumeni syndrome cells [56].

Telomeres are maintained in human tumors by activation and by alternative lengthening of telomeres (ALT) [29, 33, 105, 125, 275]. Most human tumors maintain telomeres by activating telomerase. However, in appendicular osteosarcoma ALT occurs at a higher frequency than in other types of tumors [9, 204, 255, 272, 296]. Absence of telomerase activation or presence of ALT correlates with a favorable prognosis in osteosarcoma [138, 252, 272, 296]. The ALT and telomerase-dependent mechanisms serve the same end, but they are not equivalent. Telomerase-dependent osteosarcoma cell lines have short telomeres with a minimum range of length, whereas ALT-dependent osteosarcoma cell lines have telomeres that are long, but vary in length. ALT-positive cell lines also have greater genetic instability and more translocations than the telomerase-positive cell lines [255].

One function of telomere maintenance is in stem cells. A controversial hypothesis proposed that cancers have stem cell-like subpopulations and that it is these self-renewing cells that drive tumor proliferation [244]. Stem cell-like cells have been identified in osteosarcoma tumors [70]. These cells express activated STAT3, OCT3/4, and NANOG, all of which are marker genes for pluripotent embryonic stem cells [28].

2.17 Comparative Genomic Hybridization

Since the completion of the sequencing of the human genome, efforts have accelerated to examine chromosomal abnormalities including large-scale amplifications, deletions, and variations in the number of copies in various types of cancer. This work has been catapulted by the availability of high-throughput array-based technologies that can scan the entire genome with high resolution. Comparative genome hybridization has utilized arrayed BAC or oligonucleotide probes to detect genotype variation [61]. By means of comparative genome hybridization analysis, the chromosomal instability phenotype of osteosarcoma tumors has been confirmed, with many chromosomal alterations in each tumor [8, 15, 49, 62, 103, 133, 156,

165, 174, 217, 225, 258, 260, 273, 277, 285–287, 335, 336].

Notwithstanding much variation in these analyses, some common chromosomal gains were observed for 1p, 5p, 6p, 8q, and 17p. Common chromosomal losses were observed for 2q, 10p, 14q, 15q, and 16p. Common amplification regions were observed for 1q21–q22, 1p34–p36, 5p13–p15, 6p12–21, 12q12–q14, and Xp11.2. The most common amplifications detected involved two chromosomal regions: 8q23–q24 and 17p11.2–p12. The only common deletion observed was 18q21–q22. In all cases, amplifications outnumbered deletions.

The 8q23–q24 region of amplification includes the MYC gene, as well as the TNFRSF11B, COL14A1, COL22A1, and RECQL4 genes (Fig. 2.6). The 17p11.2–p12 region contains the TNFRSF13B, MAP2K4, MAPK7 genes and TOP3A genes (Fig. 2.7). The latter forms a complex with the BLM gene, which regulates recombination in somatic cells.

2.18 Microarray Analysis of Osteosarcoma

Even though the identification of genetic alterations in osteosarcoma has progressed steadily, no single molecular marker has greater prognostic significance in osteosarcoma treatment than the current clinical markers. Clearly more comprehensive analytical technologies are needed to develop more informative classification systems and to identify new therapeutic targets.

Gene expression analysis by oligonucleotide microarray has been increasingly utilized to analyze tumors including osteosarcoma. These arrays permit a nearly comprehensive survey of

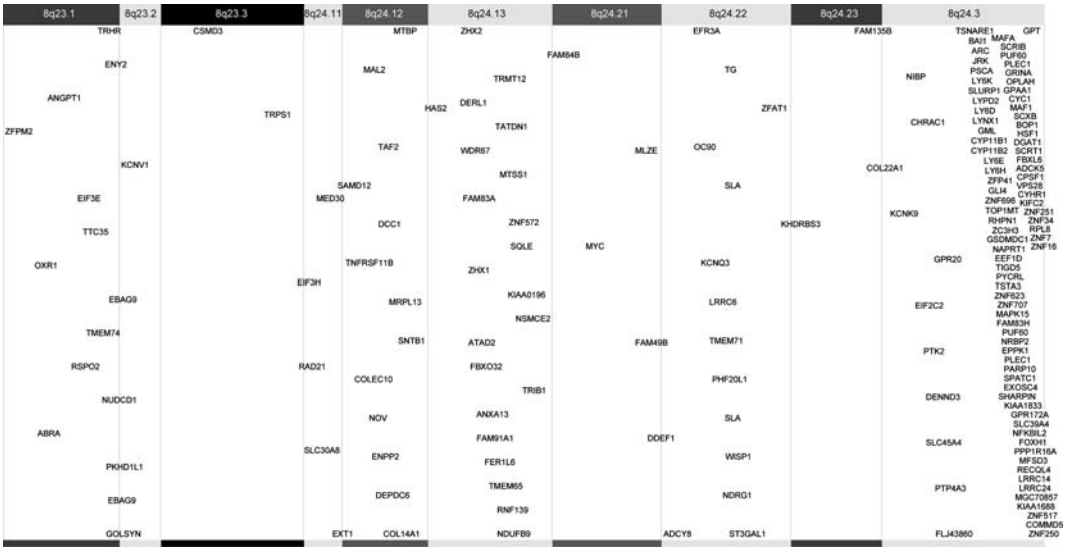

Figure 2.6. Map of the 8q23–q24 region of frequent chromosomal amplification in osteosarcoma. Map adapted from the UCSC Human Genome Browser Project [131] (http://genome.ucsc.edu/).

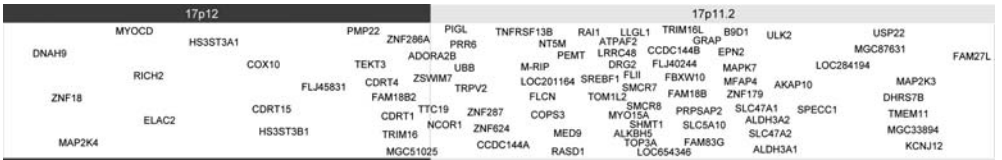

Figure 2.7. Map of the 17p11.2–p12 region of frequent chromosomal amplification in osteosarcoma. Map adapted from the UCSC Human Genome Browser Project [131] (http://genome.ucsc.edu/).

the expression patterns in the tumors, which in turn can be used to identify molecular pathways and targets for diagnosis and treatment. Microarray analysis alone can be used to develop genomic expression signatures that distinguish between outcome and therapeutic response. The method also helps divide tumors into molecularly defined categories that are associated with specific genetic pathways that can suggest novel therapeutic approaches. The use of microarrays for clinical purposes remains a challenge because of difficulties with specimen collection and their heterogeneity. In order for microarray results to be interpreted within the clinical context, they need to be validated by complimentary techniques and supported by strong bioinformatics. To reduce complexity, some microarray analyses have focused on osteosarcoma tumor cell lines [175, 194, 203, 323, 338] and mouse models [135] to identify specific known target pathways and their perturbations.

Other analyses have focused on the clinical question of identifying patients that will or will not respond to chemotherapy [173, 192, 215], thereby identifying chemotherapy-resistant pediatric osteosarcomas. Ochi et al. [215] identified a signature of 60 genes whose expression correlated with response to chemotherapy. Mintz et al. [192] identified a signature of 104 genes that correlated with response to chemotherapy. Mann et al. [176] identified a signature of 45 genes that also correlated with response to chemotherapy. Curiously, there is almost no overlap in the three gene groups. However, most genes in the three signatures groups were at high expression when there was a poor response to chemotherapy. The full significance of these findings remains uncertain. Clearly there is a need to identify a robust signature group of genes that predict response to therapy.

2.19 Summary

Osteosarcoma is a fascinating disease. Its variability in presentation, association with a number of inherited syndromes, the lack of benign precursors or other morphological determinants all make it necessary to develop molecular classification schemes for screening and identifying tumors and their likely outcome. Much progress notwithstanding, understanding of osteosarcoma remains elusive. New discoveries are therefore likely to have a profound impact on understanding the disease.

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3.

Multiple Myeloma and Other Hematological Malignancies of Bone

Rajesh Sehgal, Kristen Sanfilippo, and G. David Roodman

3.1 Multiple Myeloma and Bone Disease

Multiple Myeloma (MM) is a hematologic malignancy characterized by neoplastic clonal proliferation of plasma cells typically resulting in the presence of a monoclonal immunoglobulin (Ig) present in the blood and/or urine. MM is responsible for 1% of malignant diseases, and comprises 10% of all hematologic malignancies, making it the second most common hematologic malignancy in adults, second to only Non-Hodgkin′s lymphoma. The estimated annual incidence of MM is approximately 4 per 100,000 in Caucasians and 8 per 100,000 in African-Americans with a projected 19,900 new cases to be diagnosed in 2007 in the United States and approximately 10,790 deaths due to myeloma to occur [46]. The incidence is lower in the Asian population. The incidence of MM increases with age with median age at diagnosis being 68 years, with a greater incidence of the disease in males as compare to females.

3.2 Pathophysiology of Bone Disease in Multiple Myeloma

Bone lesions in myeloma appear to be secondary to an uncoupling of the normal bone remodeling process [4,87]. This mismatch occurs via upregulation of osteoclasts (OCL), the normal bone resorbing cells, in response to stimuli produced either directly or indirectly by malignant myeloma cells as well as decreased or absent activity of osteoblasts, the normal bone forming cells. This leads to an overall net increase in bone destruction and decreased new bone formation. Unlike other malignancies that affect the bone, even after the myeloma is treated, the bone lesions caused by the disease remain and new bone fails to form in the lesions. The pathophysiology underlying the increased osteoclast activity in MM bone disease has been widely studied and is the basis for development of new treatment modalities of the disease [35,83,93]. Decreased osteoblast activity is an important and expanding area of research.

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3.3 Osteoclast Activation/Stimulation

Increased OCL activity in myeloma is a local phenomenon. Lytic lesions only occur in close proximity to beds of myeloma tumor. The increased OCL activity is due to production or induction of Osteoclast Activating Factors (OAFs) by the myeloma cells. This occurs as a result of cell-cell interactions between myeloma cells and bone marrow stromal cells (BMSCs), via binding of VLA-4 ($\alpha_4\beta_1$ integrin on myeloma cells) to the VCAM-1 receptor expressed on the surface of BMSCs. This results in production of OAFs including; receptor activator of nuclear factor – κB ligand (RANKL), M-CSF, macrophage inflammatory protein-1alpha (MIP-1α), tumor necrosis factor-beta (TNFβ), interleukin-1beta (IL-1β), interleukin-6 (IL-6), lymphotoxin, hepatocytes growth factor (HGF), parathyroid hormone-related protein (PTHrP), matrix metalloproteinase-2,-7, and -9, and tumor necrosis factor-alpha (TNFα) [15,32,38,57,61,86,94]. No OAF has been found to be the sole cause of the increased osteoclastogenesis in myeloma, but rather it is thought to result from a combination of OAFs, which cause increased bone resorption.

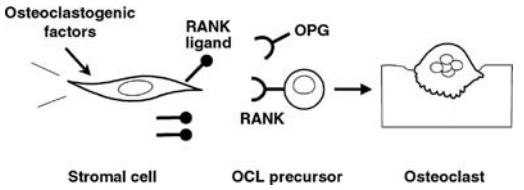

Figure 3.1. Local expression of stromal cell RANK, osteoclast (OCL) formation, and bone resorption. Locally secreted osteoclastogenic factors (e.g., cytokines and growth factors) in the bone marrow microenvironment stimulate the formation of both membrane bound and soluble RANKL by bone marrow stromal cells. After it inteacs with membrane-expressed RANK on adjacent preosteoclasts, RANKL induces OCL formation and OCL-mediated bone resorption. This process may be regulated by the local secretion of (OPG), a soluble decoy receptor that blocks the effects of RANKL. However, contact between myeloma cells and stromal cells in the bone marrow microenvironment decreases stromal cell production of OPG, thereby initiating high levels of osteolysis.

3.4 RANKL

RANKL is a member of the tumor necrosis factor gene family. It increases osteoclastogenesis by binding to the RANK receptor on osteoclast precursors to induce osteoclast differentiation and maturation. Osteoprotegerin (OPG), a naturally occurring decoy receptor for RANKL, acts as an inhibitor of RANKL, and inhibits osteoclastogenesis (Fig. 3.1). RANKL is increased and OPG is decreased in the marrow of patients with myeloma. RANKL and OPG are both normally expressed by BMSCs and osteoblasts. RANKL is increased in patients and when myeloma cells are cocultured with BMSCs, while OPG is markedly decreased [70,73]. The increase in RANKL is due to RANKL production by BMSCs, but can also be produced in low amounts by myeloma cells. These studies demonstrate that the interaction between the myeloma cells and the BMSCs increases RANKL expression; and treatment of the co-cultures with an antibody to VLA-4 (α4 integrin subunit), the molecule on myeloma cells responsible for adherence to the VCAM-1 receptor on BMSCs, or with a recombinant soluble form of VCAM-1 block the increased RANKL expression [31,70,73]. Thus, binding of the VLA-4 on myeloma cells to the VCAM-1 receptor on BMSCs is responsible, at least in part, for the overexpression of RANKL in myeloma bone marrow.

3.5 MIP-1α

Macrophage inflammatory protein–1alpha (MIP–1α) also increases OCL differentiation and maturation in myeloma [1,16,69] as well as acts as a paracrine growth factor for myeloma cells by stimulating their cell growth and survival [60]. Not only does MIP-1α stimulate OCL on its own, but it can potentiate the effects of PTHrP, RANKL, and 1,25-$(OH)_2D_3$ on OCL formation in human marrow cultures, thereby further increasing bone resorption [16] (Fig. 3.2). MIP-1α can also increase expression of VLA-4 on myeloma cells, further enhancing

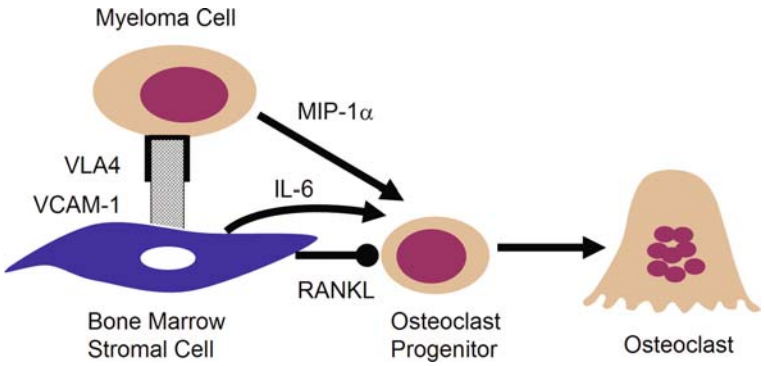

Figure 3.2. Proposed model for MIP-1α's role in myeloma bone disease. MIP-1α is produced by myeloma cells and directly stimulates OCL formation. In addition, MIP-1α enhances the effects of IL-6 and RANKL on OCL formation, upregulates VLA4 expression on myeloma cells to increase production of RANKL and IL-6 and also increases myeloma cell proliferation.

the adhesive interactions between myeloma cell and BMSCs.

3.6 Parathyroid Hormone Related Peptide (PTHrP)

PTHrP is the major mediator of the humoral hypercalcemia of malignancy [76], and elevated serum levels of PTHrP have been found in some studies of patients with myeloma and hypercalcemia, implicating PTHrP as an osteoclast activator in myeloma bone disease [45]. However, most patients with myeloma bone disease do not have elevated levels of PTHrP [27].

3.7 IL-6

Interleukin-6 (IL-6) is a growth factor and prevents apoptosis of myeloma cells. IL-6 is a potent stimulator of osteoclast formation both in vitro and in vivo [13], and IL-6 levels have been found elevated in patients with myeloma. IL-6 also enhances the effects of other factors on osteoclastogenesis such as PTHrP, IL-1 and TNFα [13]. Other stimulators of OCL formation and activity have been detected in the myeloma marrow microenvironment. These include IL3 and HGF [38,40,57], which can increase OCL formation and enhance the effects of RANKL and MIP-1α on osteoclast formation.

3.8 Osteoblast Inhibition in Myeloma

In addition to increased osteoclast activity, patients with myeloma also have decreased levels of bone formation markers such as alkaline phosphatase and osteocalcin; with marked osteoblastopenia [41]. These findings demonstrate that patients with myeloma have suppression of osteoblasts with decreased bone formation (Fig. 3.3). Physiologic differentiation and development of osteoblasts requires the transcription factor RunX2/Cbfa1 [22,28,48,50,51] in order for bone formation to occur. Phosphorylation of RunX2/Cbfa1 in bone marrow stromal cells and osteoblast precursors induces maturation of osteoblasts and expression of the osteoblast markers; collagen I, alkaline phosphatase, and osteocalcin. In one study, co-culture of myeloma cells with human osteoprogenitor cells inhibited osteoblast differentiation, with decreased numbers of early osteoblast precursors, fibroblast colony forming units (CFU-F), and osteoblast colony forming units (CFU-OB) as well as markers of osteoblast differentiation, ALP, osteocalcin, and collagen I [33]. These studies found that the activity of RunX2/Cbfa1 was decreased in these co-cultures, demonstrating that inhibition of RunX2/Cbfa1activity is responsible, at least in part, for the inhibition of osteoblast formation and maturation. RunX2/Cbfa1 has also been found to induce OPG expression, a potent

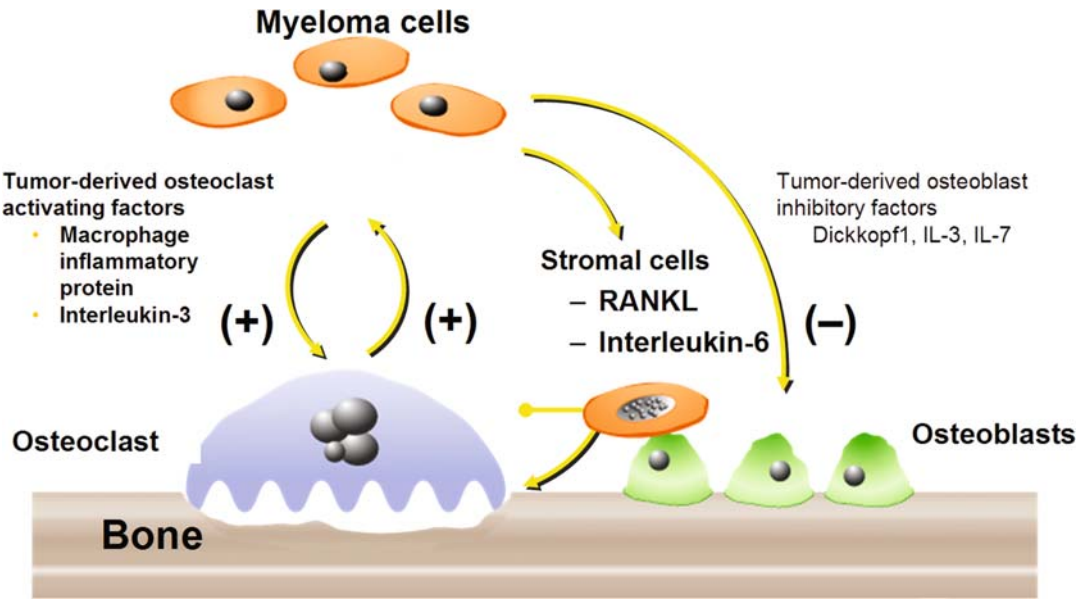

Figure 3.3. Mechanisms responsible for myeloma bone disease. Myeloma cells produce factors that directly or indirectly activate osteoclasts such as MIP-1α and IL-3. In addition, they induce RANK ligand and IL-6 production by marrow stromal cells to enhance osteoclast formation. The bone destructive process releases growth factors that increase the growth of myeloma cells, further exacerbating the osteolytic process. Myeloma cells also produce DKK1, IL-3, soluble frizzle-related protein-2, and IL-7, which suppress osteoblast differentiation and new bone formation.

inhibitor of osteoclasts. Thus, inhibition of Runx2/Cbfa1 in myeloma can further increase bone resorption [89]. Inhibition of RunX2/Cbfa1 activity in myeloma appears to be mediated by cell-cell interactions between myeloma cells and osteoprogenitor cells. These interactions are mediated by VLA-4 on myeloma cells, which binds to VCAM-1 present on osteoprogenitor cells [33].

3.9 IL-7 and IL-3

While numerous OAFs have been identified as causative factors for OCL stimulation, cytokines responsible for decreased activity of osteoblasts are not completely known. Recently, several other cytokines have been reported, which may contribute to the inhibition of osteoblast activity in myeloma. IL-7 levels are elevated in marrow supernatants from patients with myeloma when compared to healthy controls [34]. IL-7 can decrease CFU-F and CFU-OB formation in human marrow cultures, as well as decrease Runx2 activity. Further, when IL-7 blocking antibodies to marrow plasma from myeloma patients, they blunted the inhibitory effect of the marrow plasma on osteoblasts [33].

IL-3 has been found to indirectly inhibit osteoblast differentiation in myeloma via CD45+/CD116+ monocyte/macrophages [24]. This inhibitory effect was not mediated by TNFα. IL-3 is elevated in approximately 70% of marrow samples in patients with myeloma. IL-3 has also been shown to increase osteoclast formation in myeloma.

3.10 DKK1

Recently, Dickkopf-1 (DKK1) has been identified as a potential mediator of osteoblast suppression in myeloma [36,90]. DKK1 is an inhibitor of the Wnt signaling pathway, an important pathway in early osteoblast formation, and thus inhibits osteoblast precursors from forming osteoblasts.

DKK1 also acts to enhance OCL formation and bone resorption by blocking osteoblast differentiation. Immature osteoblasts and stromal cells express RANKL while more differentiated osteoblasts express OPG.

Murine models of myeloma treated with antibodies against DKK1 demonstrated a decrease in bone resorption and increased bone formation due to decreased osteoblast inhibition, and decreased tumor mass [92]. In addition to DKK1, another inhibitor of the WNT signaling pathway, soluble frizzle-related protein-2 (sFRP2) [67] has also been implicated in the osteoblast inhibition in myeloma.

3.11 Clinical Manifestations of Myeloma

Approximately 80% of MM patients manifest bone involvement, so that bone destruction is the hallmark of the disease. With bone being the primary site of disease, it is no surprise that roughly two-thirds of patients experience bone pain at diagnosis and 60% of patients suffer a pathologic skeletal fracture during the course of their disease [56,62,75]. Other clinical manifestations of the disease include renal involvement, hypercalcemia, anemia, and infections.

3.12 Bone Destruction

Bone destruction in MM can cause significant clinical morbidity. A study of over 250 myeloma patients found that the areas of bone most likely to be involved include the spine (49%), skull (35%), pelvis (34%), ribs (33%), humeri (22%), femora (13%) and mandible (10%) [53]. The most common radiographic findings of bone involvement include osteolysis, osteopenia, pathologic fractures, or a combination of the above. Eighty percent of patients experience bone pain. Bone pain typically presents in the back or chest and is exacerbated by movement and is less intense at nighttime.

3.13 Hypercalcemia

Hypercalcemia occurs in approximately 15% of myeloma patients. Causes of hypercalcemia in myeloma include increased bone resorption, decreased bone formation, impaired renal function, and in a minority of patients, increased levels of PTHrP. In contrast to humoral hypercalcemia of malignancy, hypercalcemia of myeloma is more often secondary to widespread bone involvement and renal impairment as opposed to elevated levels of PTHrP. The level of hypercalcemia in myeloma correlates with tumor burden and does not correlate with serum PTHrP levels [68]. The uncoupling of normal bone resorption/formation favors bone resorption in myeloma resulting in an overall net efflux of calcium into the extracellular fluid. Signs of symptoms of hypercalcemia include dry mouth, anorexia, renal stones, confusion, depression, nausea, vomiting, polydypsia, and polyuria. Renal impairment in myeloma is thought to cause hypercalcemia not only by an inability to clear the excessive calcium in the serum from increased bone resorption, but there is also increased renal tubular calcium reabsorption. The etiology of the increased renal tubular calcium reabsorption remains unclear, as elevation of PTHrP is not a consistent finding among myeloma patients.

3.14 Neurologic Symptoms

The most common presenting neurologic complication in myeloma is radiculopathy usually of the thoracic or lumbosacral regions. The pathophysiology of this is due to expansion of the primary tumor leading to compression of the nerve or collapse of the bone. Spinal cord compression which should be treated as an oncologic emergency is seen in 5–10% of patients. Peripheral neuropathy occurs but is typically associated with amyloidosis or more commonly as a side effect of therapy.

3.15 Other Systemic Complications

Other complications associated with myeloma include anemia, thrombocytopenia, and leukopenia. Despite extensive bone marrow involvement in some patients with myeloma, thrombocytopenia and leukopenia are rare, thus this is unlikely as the primary etiology of anemia. Rather, increased production of IL-6, fas-ligand, and MIP-1α are thought to be the primary causes of anemia. Erythropoietin levels are also probably decreased secondary to increased cytokine production. Patients with IgA myeloma are more likely to develop bleeding complications as well as hyperviscosity due to the propensity for IgA immunoglobulins to associate in polymeric formations.

3.16 Diagnosis

Criteria for diagnosis of myeloma involve the classic triad of myeloma requiring [1] $>$ 10% abnormal plasma cells in the bone marrow plus either [2] osteolytic bone lesions or [3] elevation of serum and/or urine M protein to $>$ 3 g/dl or $>$ 1 g/dl respectively [52].

3.17 Evaluation of Bone Involvement

Evaluation of bone involvement should be done using conventional radiography which is superior to Technetium-99m bone scanning. Nuclear medicine scanning reflects osteoblastic activity thus underestimating the osteolytic lesions characteristic of myeloma bone disease. If conventional radiography is inconclusive or negative in the setting of high clinical suspicion for bone disease, CT without contrast or MRI may be used, both are more sensitive than conventional radiography.

Traditionally, bone surveys have been used to both diagnose lytic lesions, and monitor response to therapy of patients with myeloma bone disease. An adequate survey includes imaging x-rays of the skull, vertebral column, pelvis, and extremities (Fig. 3.4). Due to the numerous images acquired during each bone survey, the accuracy of the interpretation of the images can be limited. The limited reproducibility of bone surveys has led to use of newer modalities such as CT scan without contrast, MRI, and PET scans to evaluate the extent of myeloma one disease. In comparison trials, MRI has been shown to have greater sensitivity than plain radiographs in detecting asymptomatic bone disease [20]. In one study of 53 patients with multiple myeloma, 55% of patients with presumed normal plain radiographs had evidence of diffuse and nodular bone involvement on MRI [20]. MRI is also superior to plain radiography for staging patients with MM (37). One study evaluated 12 patients with presumed solitary plasmacytomas on bone survey found 4 patients to have additional evidence of marrow involvement on MRI evaluation thus changing their diagnosis to MM. Thus MRI imaging of the head, spine, and pelvis is recommended in all patients with a suspected diagnosis of solitary plasmacytoma to rule out any other bone lesions.

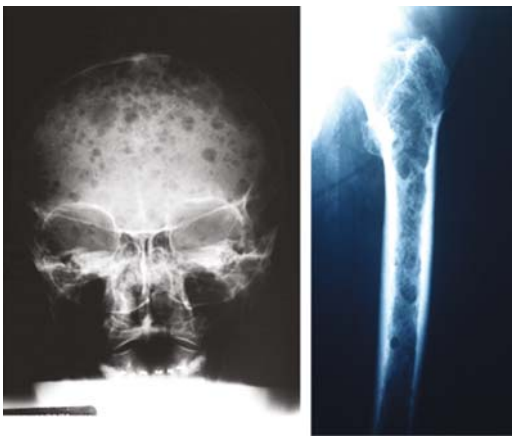

Figure 3.4. Plain film radiographs of lytic bone lesions in Multiple Myeloma. Courtesy of Dr. Mankin at the Massachusetts General Hospital.

3.18 Prognosis

While survival of multiple myeloma is improving, average length of survival even with treatment is almost 5 years with a small percentage of patients surviving for greater than 7 years. Several factors have been identified that contribute to the prognosis in MM, including karytypic abnormalities in the myeloma cells, serum levels of albumin and β2 microglobulin, which reflects tumor burden in patients without renal failure. Recently, Terpos, Evangelos et al. studied the prognostic implications of the ratio of sRANKL/OPG in myeloma and found it correlated with the extent of bone disease [88]. Terpos et al. developed a survival model including sRANKL/OPG ratio, C-reactive protein (CRP) and β2-microglobulin and compared it to the model developed by Bataille et al. using only CRP and β2-microgobulin [3,5,88]. Patients were given scores based on the value of sRANKL/OPG ratio, CRP and β2-microglobulin. The patients then were divided into 3 subgroups; low risk with a score of less than 6, intermediate risk with a score of 6–8, and high risk with a score of greater than 8 with a 5 year survival rate of 96%, 52%, and 0% respectively. Terpos et al. found that inclusion of sRANKL/OPG along with CRP and β2-microglobulin then was superior at predicting 5 year survival than Bataille's model [3, 88].

3.19 Treatment of Myeloma Bone Disease

Specific therapies aimed at ameliorating bone disease and its complications are given in conjunction with chemotherapeutic regimens to treat the underlying malignancy and include radiation therapy, kyphoplasty or vertebroplasty, surgery, and bisphosphonates.

Bisphosphonate therapy is used in patients to decrease bone pain, progression of lytic lesions, and development of new pathologic fractures and may improve survival. Bisphosphonates are synthetic analogues of pyrophosphate that act to inhibit osteoclast activity. The improvement of bone pain and life quality is thought to be due to inhibition of OCL activity mediated by induction of OCL apoptosis through inhibition of protein prenylation. Of the bisphosphonates available on the market, not all have been proved effective for the treatment of myeloma bone disease. Intravenous pamidronate, 90 mg once monthly, and zoledronate, 4 mg once monthly, are the mainstay of bisphosphonate therapy in myeloma [8]. One study which evaluated pamidronate therapy found a reduction in the number of skeletal events per patient year when compared to placebo (1.3 versus 2.2) when patients were treated for 21 months [9]. A more potent bisphosphonate being used in management of myeloma bone disease is intravenous zoledronate, a newer generation, high potency bisphosphonate. When compared with pamidronate in phase III trials, zoledronate was found at 4 mg to be as effective as pamidronate in decreasing the number of skeletal complications and need for radiation therapy, with the benefit being that it can be given over a shorter period of time (15 minutes versus 2 hours) [79]. Intravenous ibandronate is used in Europe for management of myeloma bone disease and is being evaluated for use in the United States. Oral bisphosphonates have less utility in treatment of myeloma bone disease. With oral bioavailability being less than 4%, the potency of the oral forms has a lesser benefit in decreasing the number of skeletal events when compared to the available intravenous forms [18]. Current recommendations suggest starting therapy when there is evidence of bone involvement. Duration of therapy with IV bisphosphonates is poorly understood. Optimal duration of therapy as well as interval of dosing is currently being studied. Current consensus statements recommend treating patients for 2 years and then consideration of discontinuing therapy at that time if the patient is in remission or a plateau phase of their disease [55]. ASCO guidelines currently recommend using either pamidronate or zoledronate in patients with lytic destruction of bone or spinal cord compression evidenced on imaging [54]. Patients with renal impairment should receive pamidronate over a longer infusion time.

Percutaneous vertebroplasty (PVP) is a technique that involves fluoroscopic percutaneous injection of polymethylmethacrylate (PMMA), a

component of bone cement, into vertebral bodies for stabilization or relief of pain. The diseased vertebral body is injected bilaterally or unilaterally and provides immediate relief in a significant number of patients. Kyphoplasty is a technique that involves placement of inflatable bone tamps into the vertebral body. This technique expands the vertebral body back to its original height and provides a compartment into which bone cement can be injected. Both result in decreased myeloma induced bone pain and improvement in functional activity in patients with vertebral compression fractures secondary to bone involvement [60]. Complications of the procedures are rare and include leakage of cement into surrounding tissues causing radiculopathy, spinal cord compression, and pulmonary embolism, but most often are asymptomatic [19]. Other surgical modalities are also used in managing myeloma bone disease including intramedullary nails and total hip replacement. However, the ability for surgical intervention to fail depends on the surrounding extent of bone disease and increases in correlation with time after development of pathologic fracture.

Radiation therapy is one of the earliest forms of treatment employed for management of MM. Radiation therapy now serves a main role in management of bony complications of myeloma bone disease. Myeloma is relatively radiosensitive with approximately 70% of patients with myeloma bone disease receive radiotherapy at some point during the course of their illness [12]. Patients with bone pain are typically treated with approximately 30 GY of radiation to promote bone healing and relieve pain. Higher doses of therapy are avoided because of their ability reduce or compromise future chemotherapy and prevent future autologous stem cell transplant.

As information regarding pathophysiology continues to evolve, newer treatment modalities for preventing and treating bony complications of myeloma bone disease are becoming available. One such agent is Denosumab, a monoclonal antibody to RANKL, has been studied in Phase I/II trials for management of bony complications and treatment of myeloma. Preliminary results of these studies showed that Denosumab decreased bone resorption for a total of 84 days after a single subcutaneous dose [10]. Responses were compared to pamidronate and found to be similar in activity, but with Denosumab having longer duration of inhibition of bone resorption. Final recommendations regarding Denosumab therapy await further clinical trials and analysis of current data.

3.20 Bone Involvement in Hodgkin's Disease

Osseous involvement in Hodgkin's disease (HD) occurs with a frequency of 10–15% [58]. Bone lesions in HD are often multiple and seldom seen in early stages of the disease [47]. Clinically, pain is the most common symptom from bone involvement in HD. Pain is usually deep, localized, unremitting and often nocturnal. Neurological symptoms directly due to involvement of bone have been seen in some patients. Sites of involvement include the spine, pelvis, femur, humerus, ribs, sternum, scapula and base of the skull [11,71]. However, as with non-Hodgkin's lymphoma (NHL), vertebral and femoral involvement is the most common sites affected [29]. Clinical chemistries are often not helpful in diagnosis of bone involvement. Elevation of alkaline phosphate is of hepatic origin in 90% of the cases [2]. Hypercalcemia can be seen due to excess production of $1,25(OH)_2$ vitamin D_3 or PTHrP by the lymphoma cells [27,82]. Radiographic findings include a vertebral sclerotic pattern along with periosteal reaction and hypertrophic pulmonary osteoarthropathy [29]. Bone biopsies will often show fibrosis and a mixed inflammatory infiltrate with rare atypical cells. Bone disease in patients with HD can be lytic, blastic or mixed. The mixed form of bone disease is most prevalent [7,30,44,91]. Opinion regarding prognostic significance of bone involvement in HD is divided and some investigators believe survival time in patients with bone involvement is longer [84,91]. A combined modality treatment approach is most successful in management of bone involvement HD.

3.21 Bone Involvement in Non-Hodgkin's Lymphoma (NHL)

Bone involvement is indicative of disseminated disease in NHL. Approximately 7–25% of patient with NHL will develop bone involvement during their course of disease [72]. About 4–9% of patients present with bone destruction at time of initial diagnosis [64,80]. Descriptions of bone destruction have ranged from lytic to densely osteoblastic lesions, but lytic lesions predominate [64,80]. The more highly aggressive, poorly differentiated the lymphoma the more lytic the bone metastases with little or no sclerosis. NHL has a predilection for the axial skeleton with about 75% of bone involvement in NHL being in the axial skeletal [26]. The plexus as described by Batson [6] provides an unobstructed pathway to the spine for metastases from abdominal and pelvic neoplasia. Occult lymphoma deep in pelvic nodes may spread to the axial skeleton via this mechanism before becoming detectable at the primary site. The most primitive, aggressive lymphomas are most likely to have bone dissemination. Overall, patients with diffuse rather than nodular patterns of involvement more frequently have lytic lesions. As a screening procedure for bone involvement, conventional skeletal radiography yields a high rate of false negative results, particularly when compared to technectium-99m bone scans [14].

3.22 Adult T Cell Leukemia/Lymphoma (ATL) Bone Disease

ATL is an aggressive peripheral T cell neoplasm of CD4+ T-cells associated with infection by the human T-lymphotropic virus, type 1 (HTLV-1). The disease was first noted in Japan where infection with the HTLV-1 virus affects approximately 1.2 million people, with an annual incidence of ATL being 700 persons [85]. The cumulative risk of developing ATL in patients harboring HTLV-1 is approximately 2.5% over 70 years.

3.23 Hypercalcemia Associated with ATL

Approximately 70% of patients with Adult T-cell leukemia/lymphoma (ATL) associated with human T-cell leukemia/lymphoma virus-1 (HTLV-1) develop hypercalcemia at some point during the course of their disease [49]. Hypercalcemia is a major cause of morbidity and mortality in patients with ATL [74]. As with myeloma bone disease, this is related, at least in part, to increased osteoclast activity by osteoclast activating factors produced by the tumor cells. While hypercalcemia of ATL is multifactorial in etiology, humoral hypercalcemia of malignancy seems to be the main mechanism by which hypercalcemia occurs in ATL. Several studies found that a significant number of patients with ATL had low phosphate levels, hypercalcemia, and low levels of 1,25-$(OH)_2$ D_3. These laboratory values were consistent with humoral hypercalcemia of malignancy and are secondary to increased production of PTHrP in ATL. The increased production of PTHrP in ATL is thought to occur secondary to transactivation of the PTHrP gene by the HTLV-1 tax protein. However, increased transactivation of the PTHrP gene has also been shown to occur independently of the presence of the tax protein, and thus other mechanisms likely contribute to the increased PTHrP found with ATL [42,76]. PTHrP is a protein that resembles parathyroid hormone (PTH) in its aminoterminal sequence, and like PTH, binds to the type I PTH receptor. PTHrP in turn stimulate RANKL formation thus promoting differentiation and maturation of osteoclasts. The increased osteoclasts formation leads to increased bone resorption and increased release of calcium. PTHrP also acts on the kidney to increase distal tubular reabsorption of calcium, further increasing serum calcium levels. This increased reabsorption of calcium by the kidney is thought to be the primary mechanism by which PTHrP induces hypercalcemia of malignancy [77]. Despite increased bone resorption, serum phosphate levels are also low, because PTHrP inhibits proximal tubular reabsorption of phosphate in the kidney. Thus, the

etiology of hypercalcemia in ATL is multifactorial and in different patients is likely due to a combination of increased bone resorption and increased production of PTHrP by the malignant cells.

3.24 Pathophysiology of ATL Bone Disease

In contrast to multiple myeloma patients fewer than 10% of patients with ATL develop lytic bone lesions [78]. However, bone marrow examination of ATL patients at autopsy has shown an increase in activity of osteoclasts with increased bone resorption. The pathophysiology of the increased bone resorption in ATL patients is similar to that of patients with myeloma bone disease [81], with cytokines or factors secreted by the lymphoid tumor cells or bone marrow stromal cells responsible for increasing the activity of osteoclasts (OCLs). The factors associated with increased OCL activity in ATL include; IL-1, IL-6, TNF-α/β, and MIP-1α/MIP-1β, which can increase bone resorption [66]. MIP-1α has been reported to increase bone resorption by stimulating production of osteoclastogenic factors such as IL-6, RANKL, and PTHrP by osteoblasts and BMSCs in patients with ATL [39]. RANKL has also been found to be responsible for increasing OCL activity and thus increasing bone resorption in ATL. One study analyzed the gene expression profiles of patients with ATL with and without hypercalcemia [65]. This study found that patients with hypercalcemia overexpressed RANKL and those without hypercalcemia did not. RANKL induces differentiation of precursor cells into osteoclasts in the presence of M-CSF. Lastly, IL-1, $1,25(OH)_2$ D3 and PTHrP have been reported to be elevated in patients with ATL and have been associated with increased osteoclast activity and increased bone resorption in vitro [78]. Thus, cytokines produced by the lymphoid tumor cells in patients with ATL, either directly or indirectly, enhance osteoclast stimulation and increased bone resorption.

3.25 Treatment of ATL Bone Disease

Unlike myeloma, the main bone involvement in ATL appears to be secondary to increased activation of osteoclasts, thus increasing bone resorption, with only a minority of patients experiencing osteolytic bone lesions. Thus the mainstay of treatment of bone involvement in ATL is management of the underlying disease and decreasing tumor burden. Hypercalcemia associated with ATL is also managed by treating the underlying disease in an effort to decrease tumor burden as well as the use of intravenous bisphosphonates.

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4.

Mechanism of Metastasis to Bone: The Role of Bone Marrow Endothelium

Carlton R. Cooper, Robert A. Satcher, Lisa A. Gurski, and Kenneth L. van Golen

4.1 Introduction

Cancer cells in the bone come from two sources: primary tumors or metastases from other tissues. Primary cancer of the bone is rare, with 2,370 new cases and 1,330 deaths expected in 2007. Primary cancer accounts for less than 0.2% of all cancers combined [American Cancer Society (ACS)]. The most common type of primary bone cancer is osteosarcoma (35% of bone cancer cases), followed by chondrosarcoma (26%) and Ewing tumor (16%). Most cancer cells in the bone marrow result from metastasis to bone. Breast and prostate cancers are adult cancers that frequently metastasize to bone [25]. Neuroblastoma, a cancer common in children, also frequently metastasizes to skeleton [102]. After lung and liver, the skeleton is the third most common site for cancer metastases. Of sites within the skeletal system, the spine is the most common cancer site, followed by the pelvis, hip, femur, and the skull [ACS].

Bone metastasis is significantly associated with debilitating and costly complications, termed skeletal-related events (SREs) [19, 64]. SREs include intense bone pain, pathologic fractures, and spinal cord compression. The quality of life of patients experiencing SRE is greatly diminished, with relief provided only by supportive therapies.

Bone marrow endothelial cells (BMECs) contribute to the metastatic process in two ways: (1) by docking and locking circulating cancer cells and (2) by promoting their growth in the bone marrow by forming a vascular canal (angiogenesis) that supports tumor expansion and progression [27, 81]. This chapter will address the salient features of BMECs in normal bone physiology and their role in metastasis, and suggest therapeutic approaches for inhibiting cancer cell metastasis.

4.2 The Clinical Sequelae of Bone Metastasis

Bone metastases occur in up to 70% of patients with advanced prostate or breast cancer [19, 21, 88]. These two cancers account for almost 80% of the cases of metastatic bone disease [21]. The remainder are due to carcinoma of the lung, colon, stomach, bladder, uterus, rectum, thyroid, or kidney [88, 22]. Overall, about 350,000 people with bone metastasis die annually in the United States [88]. Once bone has been invaded,

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the causative tumor tends to become incurable. Only 20% of breast cancer patients and 25% of prostate cancer patients are still alive, 5 years after their bone metastases have been diagnosed [88].

Osteolytic bone metastases cause severe bone pain, pathologic fractures, hypercalcemia, spinal cord, and other nerve compression or entrapment syndromes. Osteoblastic bone metastases cause bone pain and pathologic fractures, often because the bone produced is of inferior quality.

4.3 Natural History

Radiographs, bone scanning, and biochemical tests are used to detect bone cancer, with disease progression best estimated by combining imaging test results with bone-specific marker measurements. The rate of bone resorption correlates strongly with clinical outcome. For prostate and breast cancer patients, the median survival time is 2–3 years, whereas with advanced lung cancer and melanoma, it is usually 4–6 months [64].

From a clinical viewpoint, bone metastases are categorized as osteolytic, osteoblastic, or mixed. However, metastatic bone cancers are better described as having resulted from dysregulation of the normal bone remodeling process [88, 64]. Patients tend to have primarily osteolytic or osteoblastic metastases; they also may have mixed lesions containing both osteolytic and osteoblastic constituents. Lytic metastases are most common in multiple myeloma, melanoma, breast, lung, thyroid, renal, and gastrointestinal malignancies. The lesions in prostate cancer are predominantly osteoblastic; however, there is also increased bone resorption. Breast, lung, carcinoid, and medulloblastoma tumors also can cause sclerotic lesions [21].

Why tumors tend to metastasize to bone is not understood fully. Blood flow may be a major factor. For example, because bone metastases commonly affect the axial skeleton, it may be the type of circulation within the bone marrow space, with its sluggish blood flow, that allows metastases to establish themselves. Another contributing factor may be the interaction between metastasizing cells and the bone microenvironment. Establishing metastatic bone cancers sequentially involves primary tumor growth, the release of tumor cells into lymphatic and/or blood vessels, survival of tumor cells in the circulation, arrest in the microvasculature of the target organ, extravasation of tumor cells, invasion of the target organ, and growth at the metastatic site [88]. Malignant cells secrete factors that directly and indirectly stimulate osteoclastic activity, including prostaglandin-E, a variety of cytokines, and growth factors such as transforming growth factors alpha and beta, epidermal growth factor, tumor necrosis factor (TNF), and interleukin-1 (IL-1), IL-6, IL-8, and IL-11 [19, 21]. Local paracrine factors such as procathepsin D and RANKL, and systemic factors such as parathyroid hormone-related peptide (PTHrP) are also important [88]. Bone-derived growth factors and cytokines from resorbing bone also can attract cancer cells and facilitate their growth and proliferation. More recently, there has been a focus on the role of the bone microenvironment in supporting metastasis formation. Tumor cell interactions with bone include exchange and response to the extracellular matrix (ECM) and secreted cellular constituents. The 'vicious cycle' hypothesis states that there are complex and multiple steps in the process of bone metastasis and that a reciprocal soluble interaction occurs between breast cancer cells and the bone microenvironment which involves various cytokines, growth factors, and cellular signals[88]. More recently, it has been shown that cell–cell contact between osteoblasts and bone marrow stromal cells is an essential step in metastasis of prostate cancer to bone cancer [92, 7]. Together these heterotypic cellular interactions lead to tumor growth and alter bone physiology.

4.4 Prognosis and Clinical Course

The important prognostic factors in prostate cancer, myeloma, and breast cancer are listed in Table 4.1 [19, 21]. Skeletal metastases are the cause of severe morbidity and mortality in many cancer patients. Skeletal complications

Table 4.1. Prognostic factors in metastatic bone disease [17, 18]

Prostate	Skeletal distribution
	Performance status
	Extraosseous disease
	Alkaline phosphatase
	Hemoglobin
	PSA increase
Breast	Extraosseous disease
	Disease-free interval
	Performance status
	Estrogen receptor status
	Age
	Histologic grade
Myeloma	β2-microglobulin
	Proliferative activity
	C-reactive protein
	Immunologic phenotype
	LDH
	Serum creatinine
	Hypercalcemia

(also known as skeletal-related events) are a frequent occurrence. On average, a patient with metastatic disease will have a SRE every 3–6 months [19]. Events typically cluster around periods of progression, becoming more frequent as the disease becomes more extensive. The most common skeletal complications include pain, impaired mobility, hypercalcemia, pathologic fracture, and spinal cord or nerve root compression [19, 20, 22, 64, 1]. The clinical consequences of metastatic bone disease are greater when vertebral fractures, pain, and radiotherapy treatments are taken into account.

4.5 Hypercalcemia

Hypercalcemia is the most common metabolic complication of metastatic bone disease [19, 21, 22]. The signs and symptoms of hypercalcemia are nonspecific. The morbidity associated with moderate-to-severe hypercalcemia (serum calcium > 3 mM) includes unpleasant symptoms related to dysfunction of the gastrointestinal tract, kidneys, and central nervous system. With higher serum calcium levels, renal function and the level of consciousness deteriorate; death can follow as the result of cardiac arrhythmias and acute renal failure. Hypercalcemia occurs most frequently in cases of squamous cell carcinomas of the lung, adenocarcinoma of the breast and kidney, multiple myeloma, and lymphoma [19, 21, 22]. Hypercalcemia is generally accompanied by low parathyroid hormone levels and a high level of osteoclastic bone resorption. In cases of myeloma or breast cancer, bone resorption is typically multifocal [22].

4.6 Pathologic Fracture

For patients with skeletal metastases, pathologic fractures can have a devastating impact on prognosis and quality of life. Orthopaedic surgeons tend to treat these patients by focusing on maintaining patient mobility and treating pain. At times, surgery may be deemed too risky, but surgical intervention is required for fractures, because pathologic fractures will not heal by immobilization in a cast [44, 1, 10].

The incidence of pathologic fractures in patients with bone metastases, although uncertain [19], has been estimated to be about 60% in breast, kidney, lung, thyroid, and prostate cancer patients. Vertebral collapse is probably underreported in most series [21]. The probability of developing a pathologic fracture increases as the metastatic process progresses, and pathologic fractures are common in patients with a relatively good prognosis. Rib fractures and vertebral collapse are the most common, resulting in restrictive lung disease and kyphoscoliosis. Long bone fractures typically occur through lytic metastases in weight-bearing bones such as the femur. Damage to either cortical or trabecular bone can lead to mechanical compromise. Several scoring systems have been proposed to predict fracture, based on the site, nature, size, and symptoms [21, 1]. However, no scoring system has been universally adapted, so that the decision for surgical intervention remains with the treating physician [21, 1, 10]. Occurrence of a pathologic long bone fracture in a patient with known metastatic bone disease can be prevented by appropriate clinical management, such as prophylactic fixation of an impending fracture.

Pathologic fractures do not necessarily indicate terminal disease. It is easier to stabilize a bone before it fractures, so internal fixation

should be performed to prevent subsequent fracture. The guiding strategy for surgical stabilization is to assess the entire length of the affected bone with radiographic studies prior to surgery in order to ensure that other lesions within the bone are recognized. All potential areas for fracture in the bone should be stabilized and included in the radiotherapy field. Prophylactic radiotherapy after surgical fixation inhibits further tumor growth and bone destruction [44, 1, 10].

Primary internal stabilization followed by radiotherapy is usually the treatment of choice for pathologic fractures [21]. With tumors that tend to be radioinsensitive and/or highly vascular, such as renal cell and thyroid carcinoma, additional adjunct treatments such as local curettage and preoperative embolization may need to be used [10]. Endocrine treatment or chemotherapy also may be appropriate.

4.7 Bone Pain

How bone pain is generated is not well understood. Bone pain is thought to be the result of events such as osteolysis, the effect of growth factors and cytokines released by the tumor, the result of direct infiltration of nerves, and tissue production of endothelins [21, 22, 64]. Not all bone metastases cause pain. In one study, ˜80% of patients with advanced breast cancer developed osteolytic bone metastases, but only one-third of these patients had bone pain [22]. Pain from bone metastases has been described as a sensation of deep drilling that may be accompanied by episodes of sharp, stabbing discomfort. Night pain is frequent, persistent, and is not helped by sleep. Bone pain may be poorly localized. When due to bony instability, the pain will not be relieved by systemic treatments or radiotherapy, but requires surgical stabilization [22].

Pain management is an important component of clinical management. Good pain relief can generally be obtained if the WHO protocol for oral analgesic medications is followed [64]. However, bone pain often is undertreated [64]. The most frequent reasons cited are that the physician has underestimated the patient's pain, the patient is reluctant to report pain, or the pain has been inadequately assessed.

4.8 Spinal Cord Compression

Because spinal cord compression constitutes a medical emergency, if a patient with bone metastasis develops back pain, he/she should be evaluated and assessed for symptoms of cord compression [22, 1]. These include radicular pain radiating down a limb or around the chest, weakness or paralysis, numbness, urinary retention, incontinence, and impotence. Positive findings should prompt MRI or equivalent evaluation. If diagnosis is early, followed rapidly by treatment, rehabilitation can be successful. Treatment includes high-dose corticosteroid, surgical decompression, spinal stabilization, and radiotherapy. If spinal compression is not relieved within 24–48 h of the onset of symptoms, neurological recovery is not likely [21].

4.9 Costs of Treatment

The costs associated with the clinical aspects of metastatic bone cancer are substantial. In the US, hospital costs for cancer patients have been estimated to average $2000 per month (in 1990 dollars), with skeletal complications accounting for 63% of the hospital expenditures. In the UK, the average hospital cost of treating a patient with advanced breast cancer is ˜$10,000 (in 1990 dollars), with the largest part going to the treatment of complications of metastatic bone disease [19]. With 1.4 million new cases of cancer diagnosed in the US in 2006 [1], strategies to reduce the incidence of bone metastases and palliate established disease assume great clinical and economic importance.

4.10 Endothelial Cells: A Brief Overview

William C. Aird [3, 2] published an outstanding and comprehensive review on the phenotypic heterogeneity of the endothelium. Part 1 of the

review covers structural and functional diversity of endothelial cells and the evolutionary mechanisms underlying their diversity. Part 2 covers the unique structural and molecular features of organ-specific endothelial cells in the normal state and in diseases of the heart, lungs, liver, and kidneys. Below, we present a brief summary of relevant information on endothelial cell biology and diversity. The interested reader is urged to consult the articles by Aird for more details.

According to Aird [2], Marcello Malpighi was the first to view blood capillaries in 1661. The term endothelium was used by the Swiss anatomist Wilhelm His in 1865 to distinguish the inner lining of body cavities from epithelium. Electron microscopic studies revealed the structural diversity of various organ-specific microvascular vessels. Three endothelium structures were identified: continuous, fenestrated, and discontinuous. Endothelial cells differ in structure and function, as stated by L Florey in 1966 (reviewed in [2]): "Now it is recognized that there are many kinds of endothelial cells, which differ from one another substantially in structure, and to some extent in function."

In 1973/1974, Jaffe and colleagues, along with Gimbrone and colleagues [51, 41], reported the isolation of human umbilical vein endothelial cells (HUVECs). This cell line is commonly used as an experimental model, including studies of cancer metastasis and inflammation. Nevertheless different vascular beds express different proteins, an indication that endothelial cells are molecularly diverse [59, 79, 66]. This prompted Auerbach and colleagues [2] to state: "The concept that vascular endothelial cells are not all alike is not a new one to either morphologists or physiologists. Yet laboratory experiments almost always use endothelial cells from large vessels such as HUVEC or bovine dorsal aorta, since these are easy to obtain and can be readily isolated and grown in culture. The tacit assumption has been that the basic properties of all endothelial cells are similar enough to warrant the use of the cells as *in vitro* correlates of endothelial cell activities *in vivo*."

Since this statement, findings have been published that support the molecular diversity of endothelial cells. One study reported a difference in the HUVEC and human dermal microvascular endothelial cells (HDMVECs) response to protein kinase C activation [66]. Global expression profiling showed that endothelial cells from different blood vessels and microvascular endothelial cells from different tissues display unique expression profiles [17]. Chi et al. [17] further reported that large vessel endothelial cells and microvascular endothelial cells differed in gene expression. This also was true for arterial and venous endothelial cells. For example, a recent investigation on lung endothelial cell junctions showed that N-cadherin and activated leukocyte cell adhesion molecule (ALCAM) are preferentially expressed in the pulmonary microvascular network, as compared to endothelial cells in the pulmonary arterial network [75]. Endothelial cell diversity is of benefit to the various organs in which these cells are sited, but also represents a challenge for therapeutic angiogenesis. Just as endothelial cells differ in response to a variety of biologic agents, they are likely to differ in response to angiogenesis therapy [23].

Endothelial cells derived from the bone microenvironment are structurally and molecularly distinct from those in other organs and from large vessel endothelial cells. Schweitzer and colleagues [93] reported in 1997 that hematopoietic progenitor cells (HPCs) adhered more to primary and immortalized BMECs treated with TNF-α as compared to TNF-α-stimulated HUVEC. In addition, the importance of vascular cell adhesion molecule 1 (VCaM1) and intracellular adhesion molecule (ICaM1) in HPC adhesion to HUVEC was reduced, compared to BMECs. Also, heparin sulfate derived from BMECs has more O-sulfation of the N-sulfated domains compared to HUVEC; this unique feature contributes to the ability of BMECs to preferentially bind to the stromal derived factor-1 (SDF-1) [71]. The chemokine SDF-1 present on BMECs is presented to HPC to mediate their homing to the bone marrow [72].

Interestingly, endothelial cells in different sites within the bone microenvironment are not alike. Using a mouse model, Sosnoski and Gay [98] demonstrated differences between genes expressed in bone-derived vascular endothelial cells (BVECs) and marrow-derived endothelial cells (MVECs). BVECs preferentially express

aldehyde dehydrogenase 3A1 (ALDH3A1), secreted modular calcium-2 (smoc-2), CCAAT enhancer binding protein (C/EBP-β), matrix metalloproteinase 13 (MMP-13), and annexin 8 (ANX8). MVECs preferentially express Spα and matrix GLA-protein (MGP). For a discussion of the functions of these proteins, the reader is referred to [98]. In this publication, Sosnosly and Gay [98] point out that even different vessels in the same organ are composed of distinct endothelial cells that differ in the proteins they express.

4.11 Bone Marrow Endothelial Cells and Bone Physiology

Osteogenesis, the development and maturation of osteoblast precursors, is intimately linked to bone marrow angiogenesis, as only cells situated near a blood vessel give rise to bone tissue. For example, osteoblast-derived vascular endothelial growth factor (VEGF) stimulates bone repair in a mouse model by promoting angiogenesis and osteoblast differentiation [99]. These two processes become coupled during skeletal development in mice as a result of the action of the hypoxia-inducible factor α (HIF-α). According to this report, osteoblasts sense a reduction in oxygen tension and respond by activating the HIF-α pathway by the action of which VEGF biosynthesis is increased, leading to the stimulation of angiogenesis in BMECs. The increase in blood vessel formation provides the oxygen needed for osteoblast differentiation and bone growth. In support of this model, mice that overexpress HIF-α in osteoblasts also express high levels of VEGF and develop very dense highly vascularized long bones. The reverse was observed for mice that lack HIF-α, namely, their long bones were thinner and less vascularized than in controls.

Bone resorption requires the adhesion of osteoclast precursors to BMECs so that the precursors can migrate to the site of resorption (reviewed in [12]). BMECs act to separate osteoclast precursors from the bone surface. Osteoclast precursor cells recognize specific cell surface receptors, expressed in a time- and space-dependent manner, on BMECs located in the cutting cone of cortical bone. This event is similar to the transendothelial migration (TEM) of both neutrophils and cancer cells [68].

It is apparent that BMECs are vital for normal bone physiology because, in response to osteoblast-derived VEGF, they construct a blood supply needed for bone formation and repair. In addition, BMECs direct osteoclast precursor cells to the site for bone resorption. They do so because they express specific cell surface receptors that facilitate adhesion and TEM.

4.12 Bone-Marrow Endothelial Cells in Tumor Angiogenesis

Little is known about the factors that stimulate growth and migration of BMECs during tumor-induced angiogenesis. Bone marrow angiogenesis is critical for the metastatic progression of breast cancer, multiple myeloma, and prostate cancer [81, 16, 82, 68]. Bone marrow microvessel density is significantly higher in breast cancer patients with bone disease than in patients without evidence of bone metastasis [15]. Barrett and colleagues [8] showed that conditioned media from PC-3, a prostate cancer line derived from a bone metastasis, stimulated two bone marrow-derived endothelial cell lines, HBME-1 [62] and TrHBMEC [93], to form large, branching, cord-like structures that mimic angiogenesis. This was not observed when HDMVECs were exposed to PC-3 conditioned media. Using cDNA microarray and cytokine and neutralizing antibodies, Barrett and colleagues [9] showed that TGF-β, secreted at low levels by PC-3, mediated the differentiation of HMBE-1 through TGFβRII. Moreover, angiogenesis-associated genes in bone, liver, and lymph node metastases from prostate cancer differed in the degree of angiogenesis expression [70]. Similarly, expression of bone sialoprotein and factor XIII was increased and expression of ephrin-A1 and angiopoietin-2 decreased in prostate cancer cells that had metastasized to bone, compared to prostate cancer cells that had metastasized to liver and lymph nodes. This suggests that angiogenesis is induced differently in bone, lymph node, and liver.

Bone marrow angiogenesis induced by multiple myeloma has been studied more extensively than breast- and prostate cancer-induced bone marrow angiogenesis [reviewed in [82]]. Dormancy is induced in multiple myeloma during the avascular phase (non-active multiple myeloma), whereas factors such as hypoxia and shear stress stimulate non-active multiple myeloma to switch to an angiogenic phenotype, called the vascular phase or active multiple myeloma. Factors that have been identified as producing bone marrow angiogenesis in multiple myeloma are IL-6, TNF-α, VEGF, fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF-1), insulin-like growth factor (IGF-1), macrophage inflammatory protein-1 (MIP-1), monocyte chemotactic factor-1 (MCP-1), and SDF-1. These cytokines and the cellular signals they induce may therefore constitute novel therapeutic targets for vascular phase active multiple myeloma, a condition that remains a clinical challenge.

4.13 Bone-Marrow Endothelial Cells as an Adhesive Substrate for Circulating Cancer Cells

Circulating cancer cells interact with BMECs prior to establishing a metastatic lesion. The initial transient binding of the cancer cell to BMEC is called docking, followed by a firmer attachment called locking (reviewed in [24]). PC, BC, and neuroblastoma bind preferentially to BMECs, compared to HUVECs and HDMVECs [62, 16, 102]. Rolling of a bone-metastatic prostate cancer cell line on IL-1β-stimulated bone marrow endothelium under shear flow is mediated by E-selectin [32]. E-selectin in turn binds to cognate glycoprotein ligands on prostate cancer cell lines [33]. For breast and prostate cancer cells to bind to BMECs may require endothelial lectin, galectin-3, and Thomsen–Friedenreich (TF) antigen-expressing glycoprotein on the tumor cell surface [42, 53]. Interestingly, Romanov and colleagues [87], using C4-2B of the LNCaP human prostate cancer progression model, have reported that even prostate-specific antigen (PSA) can mediate prostate cancer cell adhesion to BMECs. Cooper and colleagues [26] demonstrated that BMEC growth on bone marrow-soluble ECM extract increased the affinity of BMECs for PC-3 cells. Neuroblastoma cells that express a high level of IGF-I receptor adhered preferentially to BMECs, whereas neuroblastoma cells that lacked the IGF-I receptor, did not. This suggests that IGF-I, a common growth factor in the bone marrow, may enhance adhesion of specific cancer cells to BMECs. It is now known that SDF-1α stimulates prostate cancer cell adhesion to BMECs and that SDF-1α contributes to specialized microdomains on BMECs that are targeted by circulating malignant cells [95, 100]. Therefore, both IGF-I and SDF-1 are likely to play vital roles in regulating cancer cell adhesion to BMECs.

Invasion of the target organ during metastasis requires cycling of focal contacts and dynamic adhesive interactions with the endothelium and subendothelial matrix [94]. As discussed above, some cancer cells may dock onto endothelial cells, with the docking process mediated by endothelial selectins and cancer cell glycoproteins. Locking onto the endothelium is usually mediated by integrins [25]. In breast cancer, cross-linking of CD44, another cell surface glycoprotein known to function as the receptor for both osteopontin and hyaluronan, induces expression of α4β1 and αLβ2, leading to tumor–endothelial adhesion and TEM [106]. After stimulation by HGF and eventual release of TEM, CD44 participates directly in adhesion of breast cancer cells to BMECs. Furthermore, CD44 and α4β1 can mediate murine myeloma binding to BMECs and adhesion of prostate cancer cells to BMECs [77, 34].

How important cancer–endothelial adhesions are to enhance the ability to metastasize and for transmigration of the endothelium requires further study. As demonstrated by Sikes et al. [94], trapping and juxtaposition of a metastatically competent cancer cell to the endothelium is as important as selective adhesion. Their findings showed a marked increase in TEM by the more aggressive cancer cells. Nonetheless, there is substantial evidence of a significant role for selectins, integrins, cadherins, immunoglobulins, and other CAMs in

adhesions before TEM can proceed. Cytokines may activate both cancer and endothelial cells to mediate 'docking' and expression of CAMs during the formation of weak cancer cell adhesions to endothelium. Cytokines may strengthen these adhesive interactions to 'lock' the cancer cell to the endothelium [13, 56]. CAMs that are present at endothelial–endothelial and tumor–endothelial junctions play an important role in the cycling of focal contacts during diapedesis of the cancer cell through the endothelium.

Even though tumor adhesion to endothelium facilitates TEM, tumor–endothelial adhesion can also serve as a defense against metastasis [50]. The transmembrane protein KAI is a metastasis suppressor when present at the surface of tumor cells [7] and its expression is reduced in metastatic tumors [86]. KAI1 binds the chemokine receptor, Duffy antigen/receptor for chemokines (DARC), on endothelial cells, and the interaction of these two molecules plays a role in trapping prostate cancer cells in the capillaries of the bone marrow during tumor cell arrest to suppress metastasis [7].

4.14 Rho GTPases: A Brief Background

Many of the diverse components of the metastatic phenotype, including angiogenesis, are controlled by members of the Ras superfamily of small GTP-binding proteins. The number of members of the superfamily exceeds 130, which fall into the Ras, Rho, Arf/Sar1, and Rab/Ran subfamilies (reviewed in [101]).

All aspects of cellular motility and invasion, including cellular polarity, cytoskeletal organization, production of angiogenic factors, and transduction of signals from the outside environment are controlled by an interplay between members of the Rho-GTPase subfamily [32, 38, 54, 91, 31, 35, 6, 84, 108, 55, 90]. The Rho-GTPases subfamily consists of small monomeric GTP-binding proteins (20–30 kDa) that have been highly conserved throughout evolution [65].

Rho was isolated in 1985 from *Aplysia californica* [65] due to its homology to Ras (Rho stands for Ras homologous). Identification of three closely related human Rho homologs, RhoA, RhoB, and RhoC, soon followed [65]. Other members of the Rho subfamily, human Cdc42 (a homolog of yeast Cdc42), Rac1, Rac2, and Rac3, were found to be distinct in function from the other Rho proteins [85, 57, 73].

Since Rho was first described, 21 Rho-family genes encoding 23 signaling molecules have been identified in humans [84]. These proteins, although related, differ from one another by virtue of their structural, biochemical, and functional features (reviewed in [84, 107]). Similar to Ras, Rho proteins are localized at the inner plasma membrane by a C-terminal lipid modification. Localization to the plasma membrane is necessary for activation [48]. Also like Ras, the Rho proteins can bind GDP/GTP and hydrolyze GTP. This in turn activates downstream effector molecules and leads to a cellular response [65, 85]. Rho proteins are inactive in their GDP-bound state, but become active when GTP-bound [65, 85]. Some Rho-family members, such as RhoE/Rnd3 and RhoH, lack intrinsic GTPase activity and appear to inhibit the activity of other Rho GTPases [30, 29, 74, 63].

Of the nearly two dozen Rho genes that have been identified, four members, RhoA, RhoB, Rac1, and Cdc42, have been studied extensively. A series of elegant experiments, by Alan Hall and Anne Ridley, determined the functions of the RhoA, Rac1, and Cdc42 proteins. At nearly the same time that RhoA was identified, C3 exotransferase, a toxin derived from *Clostridium botulinum,* was found to effectively target Rho (A, B, and C) proteins, but with little effect on Rac or Cdc42 [5, 37, 84]. C3 exotransferase inhibits Rho protein activity by promoting ADP-ribosylation of asparagine 41, thereby inhibiting the interaction between GTP-bound Rho and downstream effector molecules [4]. This does not affect the active state of Rho, but the GTPase becomes ineffectual. By combining C3 exotransferase treatment and cell transfection with constitutively active forms of RhoA, the Hall and Ridley groups showed that Rho formed bundles of actin stress fibers, thereby causing the actin cytoskeleton to become organized [80, 85]. Other experiments have shown that Rac1 and Cdc42 proteins play a role in forming actin-rich lamellipodia and filopodia,

respectively [85, 73]. The dominant-negative GTPases exert their effect by entering into non-productive interactions with the guanine nucleotide exchange factors (GEFs), thereby preventing activation of the wild-type Rho proteins. The individual role played by each GTPase in bringing about cellular motility was then established. This included lamellipodia and filopodia formation, control of vesicular trafficking, apoptosis, cellular transformation, cell adhesion, growth control, and angiogenesis [43, 83, 28, 32, 39, 14, 18].

Cytokine, chemokine, growth factor, or integrin ligation activate Rho GTPases through interactions of several Rho regulatory proteins, the GTPase-activating proteins (GAPs), GDP-dissociation inhibitors (GDIs), GDI release factors (GRFs), and GEFs [78, 40, 54, 69, 90] (see Fig. 4.1). The entire cycle is balanced by GDIs, which prevent GDP dissociation and act to sequester the Rho proteins in the cytoplasm [69]. The GDP-bound, inactive Rho is liberated from the GDI by the GEF and becomes localized at the inner plasma membrane, remaining in the inactive state. Activation of the GEFs by tyrosine kinase growth factor receptors or integrin ligation dephosphorylates GTP and activates the GTPase [78]. Simultaneously, growth factor receptor-induced phosphorylation of RasGAP leads to heterodimer binding and inactivation of RhoGAP, allowing the GTPase to remain active [89]. GTP binding produces a conformational change in Rho that causes interaction with and activation of downstream effector proteins, inducing a cellular response [11]. Through unknown mechanisms, the RasGAP/RhoGAP heterodimer dissociates and the active GAPs catalyze hydrolysis of GTP to GDP, inactivating the GTPase and closing the cycle [89].

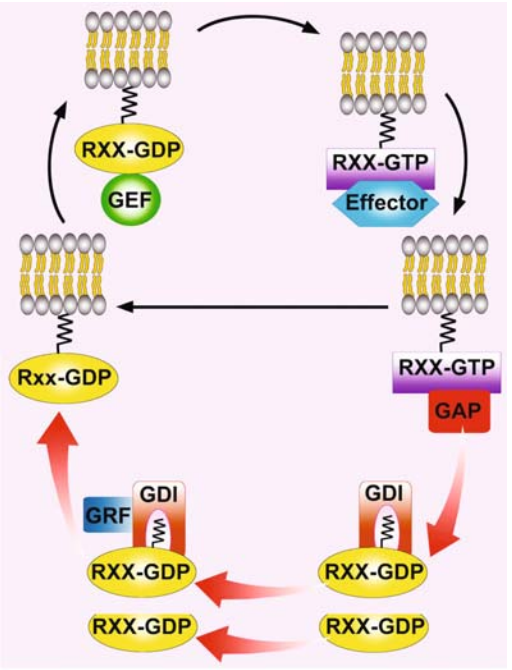

Figure 4.1. The regulation of Rho GTPase.

4.15 Rho GTPases and Angiogenesis

Early experiments had shown that Ras GTPase activation led to expression of pro-angiogenic and inflammatory cytokines. From this was inferred that Rho GTPases also modulate the production of pro-angiogenic factors [58]. The introduction of constitutively active Ras was then shown to upregulate VEGF-A production in epithelial cells and fibroblasts. Cell lines that express dominant active K- or H-Ras also display increased levels of VEGF-A, but not VEGF-B or -C. Under normoxic and hypoxic conditions, Ras mediates VEGF transcription in several ways [76, 36]. Ras signal transduction pathways activate transcription factors such as AP-2, Sp1/3, and HIF-1, and overexpresssion of the translation initiation factor, eIF-4E, upregulates VEGF. The 4E-binding protein 1 (4E-BP1) causes downregulation of eIF-4E, whereas phosphorylation of 4E-BP1 by the Ras-Raf-ERK pathway initiates increased eIF-4E translation [46, 52].

Because Ras and Rho proteins are homologous and expressed in human cancers, it seemed logical that activated Rho proteins would induce pro-angiogenic and inflammatory cytokines, which in turn would stimulate the production of angiogenic factors by autocrine or paracrine mechanisms [58]. Regulation of the transcription of acidic FGF is regulated by Ras, Rac1, and Cdc42; the latter two are members of the Rho GTPase subfamily [109]. Even though Ras

and Rac1 activate the bFGF promoter in vitro, these GTPases have not been shown to regulate bFGF/FGF-2 transcription in vivo.

When oncogenic Ras activates Rac1 through the stress-activated c-Jun kinase (JNK) pathway, transcription of thrombospondin-1 and -2 (Tsp-1 and -2) is suppressed in tumor cells [109, 58]. Thrombospondins are ECM proteins that inhibit angiogenesis [109]. If Tsp-1 and -2 transcription is repressed, VEGF production increases substantially.

RhoA GTPase is implicated in prostate cancer angiogenesis via IL-8 [47]. RhoC, a close homolog of RhoA and RhoB, induces expression of pro-angiogenic factors, IL-8, IL-6, bFGF, and VEGF-A, in a mammary epithelial cell line [103]. Addition of conditioned medium (from RhoC-transfected human mammary epithelial cells or SUM149 inflammatory breast cancer cells) has led to significant new vessel outgrowth [103]. In contrast, media that contained low levels of IL-8, IL-6, bFGF, and VEGF-A did not stimulate new vessel formation. Interestingly, when Rho activity was inhibited by C3 exotransferase, the RhoC expressing cells did not produce angiogenic factors. Inhibition of downstream targets of RhoC signaling, PI3K, MAPK, and p38, indicated that p38 had a role in RhoC-induced production of VEGF-A. Both p38 and MAPK are required for RhoC-induced migration [55, 104].

4.16 Rho-Mediated Endothelial Cell Motility

The formation of new blood vessels requires that endothelial cells migrate into the area directed chemotactically by angiogenic factors. Rho GTPases regulate cell migration through control of the cytoskeleton, with the process of migration dependent upon reorganization of the actin cytoskeleton, changes in the adhesion to the ECM, and the link between the two transmembrane proteins. For a cell to become migratory, several steps must occur: (1) cell protrusions, lamellipodia or filipodia, must be extended in a direction away from the cell, (2) new attachments to the ECM must be formed and stabilized, (3) contractile forces must pull the cell forward, typically through the action of myosin-based motors, and (4) adhesions in the rear of the cell must be released [61].

Lamellipodia and filipodia are formed when actin filaments become a meshwork and protrude through the cell membrane [73]. Rac GTPase is thought to be the principal enzyme involved in the formation of lamellipodia. When the dominant-negative Rac is incorporated in epithelial cells, the formation of lamellipodia is inhibited, whereas introduction of a constitutively active Rac leads to the formation of broad, non-directional lamellipodia in the absence of a motogenic stimulus [85]. The same applies to the regulatory role of Cdc42 with respect to the formation of filipodia.

Activation of Rac GTPase in endothelial cells must be tightly regulated for migration to be appropriately directed [96]. Activation of Rac at the proper time and site avoids the formation of broad, non-directed lamellipodia which prevent proper migration. Formation of lamellipodia and filipodia depends on actin reorganization and attachment formation, two processes that are regulated by different downstream effectors of Rac and Cdc42. Reorganization of actin is due to GTPase control of actin-associated proteins such as ARP and cofilin [67]. Attachment formation is regulated by modifying focal adhesion of FAK and paxillin [49]. A third actin structure, actin stress fibers, is regulated by RhoA GTPase [73]. Actin stress fibers are long actin polymers that stretch throughout the body of the cell and are anchored to the ECM by focal adhesions. Actin stress fibers are involved in cell contraction and in stabilization of cell adhesions. Because migration involves alternating extension/contraction and adhesion/release of the cell, Rho GTPases are bound alternatively to active and inactive GDP.

Vascular endothelial cell motility and migration are brought about largely by VEGF signaling through the VEGFR-2/KDR/Flk-1 tyrosine kinase receptor. VEGF enhances migration of HUVEC, a process that is inhibited by a dominant-negative RhoA, is introduced in the cell culture, or is treated with the Rho kinase inhibitor Y-27632 [105]. Cross-talk between VEGFR-2 and the heterotrimeric G protein, Gq/11, stimulates RhoA- and Rac-mediated

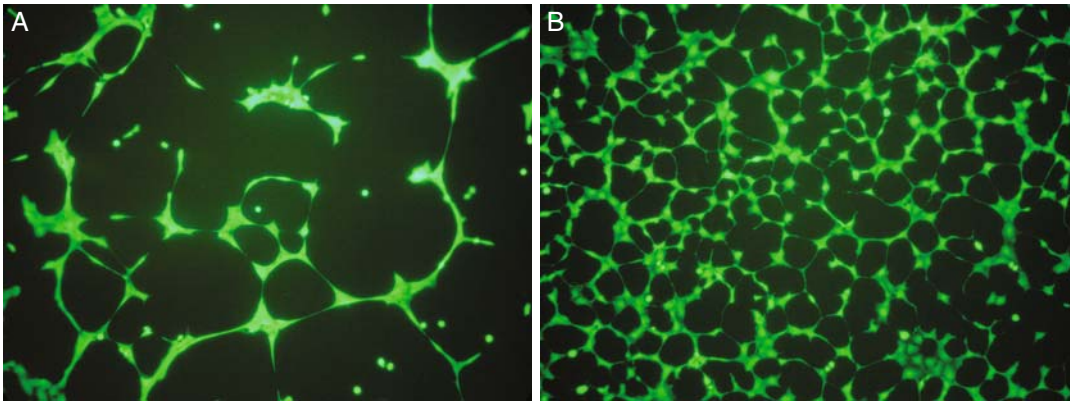

Figure 4.2. ROCK inhibitor Y-27632 stimulates BMEC tube formation. BMECs were treated with Y-27632 for the entire duration of the Matrigel® assay (18–24 h). **A**: Untreated BMECs on Matrigel® for 24 h. **B**: ROCK inhibitor (30 μM Y-27632) treated BMECs on Matrigel® for 24 h.

endothelial cell migration [110]. Activation of RhoA in this system is partially inhibited by introduction of a dominant-negative Rac. However, in cultures of microvascular endothelial cells from human foreskin, VEGF-stimulated chemotaxis was due to activation of Rac, not of RhoA [97]. Further, Cdc42 downstream from VEGFR-2 is necessary for stress fiber formation and p38-dependent endothelial cell migration [60]. It is likely, therefore, that GTPases play a significant role in endothelial cell migration.

4.17 Closing Comments and an Emerging Concept

Not much is known of how Rho GTPases function in angiogenic activity of BMECs, as most relevant studies were done with HUVECs and HDMVECs. We have preliminary findings which indicate that BMECs act differently from HDMVECs in angiogenesis. BMECs seem to be more sensitive to a novel anti-angiogenic compound than are HDMVECs. This novel compound activates RhoA GTPase and inhibits microtubule organization. However, when Rho kinase (a.k.a. ROCK), one of the downstream targets of RhoA, was inhibited, vascular tubule formation of BMECs significantly increased (Fig. 4.2). These findings contradict other reports where inhibition of ROCK decreased angiogenic activity [45]. The mechanisms of angiogenic activities, such as cell division and cell motility, therefore seem to differ in different endothelial cells. This would imply that information on one cell type (i.e., HUVECs and HDMVECs) cannot necessarily be extrapolated to other cell types (i.e., BMECs). If these differences are confirmed, therapeutic approaches for tailoring site-specific anti-angiogenic therapies targeting BMECs cannot be based on findings with HUVECs and HDMVECs.

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5.

Lysophosphatidic Acid: Role in Bone and Bone Cancer

Olivier Peyruchaud and Norman J. Karin

List of Abbreviations

μM	micromolar
ATX	Autotaxin
Ca^{2+}	Calcium
DAG	Diacylglycerol
Edg	Endothelial differentiation gene
EGF	Epithelial growth factor
ER	Endoplasmic reticulum
ERK	Extracellular receptor activated kinase
FAK	Focal adhesion kinase
GPCR	G protein-coupled receptor
IL-6 IL-8	Interleukin 6 and 8
LDL	Low-density lipoprotein
LPA	Acid lysophosphatidic
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPS	Lysophosphatidylserine
LysoPLD	Lysophospholipase D
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
nM	nanomolar
PDGF	Platelet derived growth factor
PI3K	Phosphatidyl inositol-3 kinase
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PTH	Parathyroid hormone
RANK-L	Receptor activated nuclear factor-κB ligand
ROCK	Rho-associated coiled kinase
VEGF	Vascular endothelial growth factor

5.1 Lysophosphatidic Acid

The (1- or 2-)Acyl-lyso-sn-glycero-S-phosphate (LPA) is the simplest glycerophospholipid (Fig. 5.1). LPA derives from the hydrolysis of membrane phospholipids following sequential actions of different phospholipases (PLs) such as PLC, PLA1, PLA2, and lysophospholipase D (LysoPLD/Autotaxin [ATX]) (Fig. 5.1). LPA is formed along two pathways. The first depends on PLC activity. Upon cell activation, the PLC cleaves phosphatidylinositol, releasing inositol triphosphate into the cytoplasm, whereas diacylglycerol (DAG) remains attached to the plasma membrane. DAG then undergoes phosphorylation by the DAG-kinase, to yield phosphatidic acid (PA) which in turn is cleaved by PLA1 or PLA2 into a fatty acid chain (in sn-1 or sn-2 position) and lysophosphatidic acid. Thus, the term 'LPA' refers to a family of molecules with multiple possibilities to combine the position of the carbon chain in

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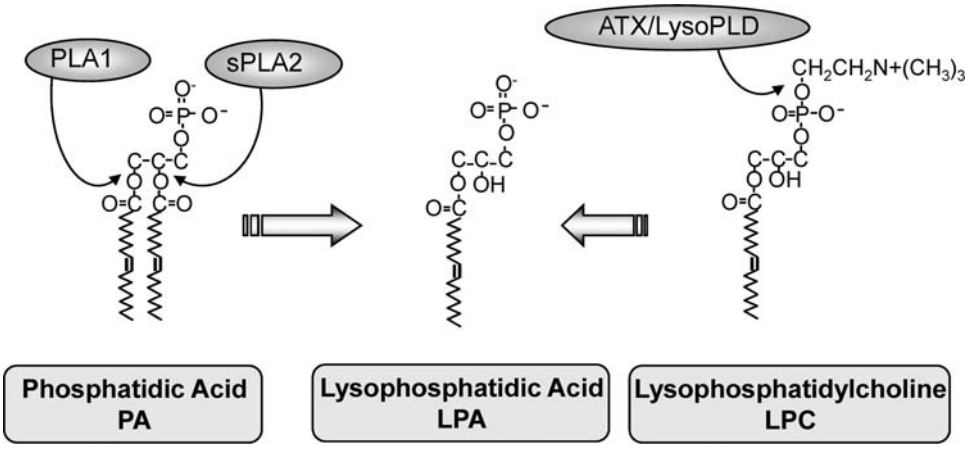

Figure 5.1. LPA formation.

sn-1 or sn-2 on the glycerol backbone and its levels of saturation. The second pathway that leads to LPA production is mediated by ATX activity. Because of its lysophospholipase D activity, ATX releases the choline moiety from lysophosphatidylcholine (LPC) to generate LPA directly (more details on ATX will be described later in this chapter).

5.2 In vivo Sources of LPA

Blood platelets are the most important source of LPA in the body, notably during platelet aggregation induced by thrombin. LPA is detected in plasma at concentrations on the order of 0.1 μM that reaches micromolar levels in serum after platelet aggregation [22]. However, local concentrations of LPA close to the site of platelet activation may be greater (5–20 μM) [33]. Boucharaba et al. [11] observed that blocking platelet function in animals results in a depletion of LPA in the blood. Aoki et al. [4] have reported that when serum is treated with thrombin, the LPA derived from platelets represents only part of the total LPA in serum. In fact, upon activation, platelets produce high amounts of LPC, lysophosphatidylethanolamine (LPE), and lysophosphatidylserine (LPS) because of the downstream actions of sPLA2 and of a specific PL for LPS. These lysophospholipids then will become the substrates for the lysoPLD/ATX activity present in the plasma and will be transformed into LPA [5]. Boucharaba et al. also found that when metastatic breast cancer cell lines were stirred with human platelets at 37°C, there occurred aggregation and production of biologically active LPA [11].

Human adipocytes express ATX [26] and produce LPA in vitro [118]. Adipocyte-derived LPA is mitogenic for preadipocytes, with the mechanism dependent upon MAP kinase activation. LPA produced by adipocytes in vivo may therefore increase the pool of fat cells and contribute to obesity [91]. Because adipocytes and preadipocytes are present in bone marrow, bone constitutes a favorable environment for LPA target cells and may be a source of LPA.

NIH3T3 fibroblasts treated with PDGF produce LPA [32]. Treatments of fibroblasts with the bacterial phospholipase D *(Streptomyces chromofuscus* phospholipase D*)* induce conventional cell signaling pathways associated with LPA stimulation (Ras, Rho, MAP kinases, DNA synthesis, calcium mobilization). Van Dijk et al. [120] have shown that LPC located in the outer layer of the plasma membrane can be a substrate for the bacterial PLD to form LPA. Therefore many cells can be the source of LPA, provided they are in the presence of exogenous PLD or LysoPLD.

The secreted PLA2 (sPLA2) has limited activity on phospholipids that are located in intact membranes, but has markedly increased activity

on damaged membranes or membrane microvesicles [29]. Microvesicles are spontaneously produced by cells undergoing apoptosis and are also generated by activated cells such as aggregated blood platelets, erythrocytes, or tumor cells. LPA accumulates in biological fluids under pathological conditions [72, 104] as in the aqueous humor after corneal injury [72], or in ascites fluids of patients with ovarian cancer [104]. Weakly oxidized LDL give rise to biologically active LPA which, in turn, has been implicated in the pathogenesis of atherosclerosis [108]. Thus LPA can be seen to exert biological activity under pathological conditions.

Even though a normal role for ATX in bone has not been reported, ATX knockout mice die as embryos; this suggests that ATX plays a crucial role in development [113, 121]. In heterozygous animals the concentrations of LPA in plasma and serum [113, 121] were half of the normal values. For more than a decade ATX has been known as a motility factor, secreted by melanoma cells [109]. Recently, the role of ATX in cell migration has been related to its lysoPLD activity and the formation of LPA [117]. ATX concentration varies in different human tissues with the highest levels in brain, placenta, ovaries, small intestine, and adipose tissue [68]. The knockout studies referred to above suggest that ATX is the principal source of LPA in the blood. Compared with corresponding normal tissues, ATX expression was found to be elevated in a wide range of cancerous tissues, i.e., colon, breast, prostate, lung, and liver [117]. Yang et al. have shown that the invasiveness of breast cancer cells correlated with elevated levels of ATX expression [133], but the mechanism is not known. In MDA-MB-435 breast cancer cells, integrin $\alpha 6\beta 4$ regulates ATX gene expression through the NFAT-1 transcription factor [16]. This finding suggests that adhesion molecules control the activity of ATX.

5.3 Biological Activities of LPA

Early in the 1960s, Vogt revealed that LPA was a bioactive lipid that causes contraction of smooth muscle cells [124]. Since then, a broad range of biological activities have been assigned to LPA (see review by Moolenaar [83]). Some activities induce rapid cell responses that are independent of protein synthesis, for example, cell contraction, mobility, chemotaxis, and invasion. At the same time, LPA stimulates gene transcription to mediate cell cycle progression and survival. It also enhances the secretion of growth factors (VEGF) and of cytokines (IL-6, IL-8) that act as secondary autocrine or paracrine factors [82]. The biological functions of LPA in bone will be discussed in more detail in the sections that follow.

5.4 LPA Receptors

Because LPA is a lipid it has the potential to bind to cell membranes hydrophobically. The existence of LPA receptors was subject to debates until, in 1985, Watson and coworkers showed that LPA activity on blood platelets was mediated through the activation of an unidentified cell surface receptor [127]. Later, LPA receptors were detected at the cell surface of fibroblasts, epithelial cells, and lymphocytes [51].

LPA receptors have been recently cloned and characterized. All present the canonical structure of seven transmembrane-spanning domains of G-protein-coupled receptors (GPCRs) (see Fig. 5.2). On the basis of sequence homologies to the Edg-1 receptor first detected in endothelial cells [69], many LPA receptors belong to the endothelial differentiation gene family (Edg). Of eight Edg receptors identified so far, three are specific for LPA (LPA_1/Edg-2, LPA_2/Edg-4, LPA_3/Edg-7, [3, 7, 44] and five recognize another bioactive lysolipid, sphingosine 1-phosphate (S1P) [4, 47, 69, 76, 119]. Recently, three other LPA receptors, LPA_4/GPR23/P2Y9, LPA_5/GPR92, and LPA_6/GPR87 [62, 87, 111], have been found to share the same GPCR structure, but have a very low sequence homology to the Edg family members. Rather, they are related to the purinergic receptors (P2Y).

LPA_1 is the most ubiquitous LPA receptor, with very high expression in placenta, brain, and heart. The expression profiles of LPA_2 and LPA_3 are more restricted. LPA_2 is

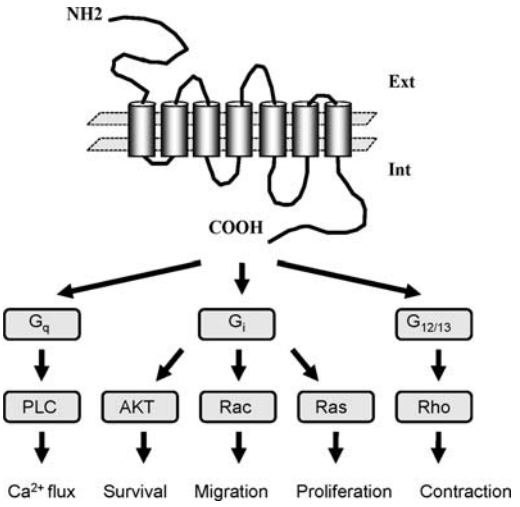

Figure 5.2. Structure of LPA receptors and cell signaling pathways activated through interaction with LPA.

expressed abundantly in the thymus and spleen [3], whereas LPA_3 is expressed primarily in the pancreas, testis, and prostate [7]. LPA_4 expression is low in human tissues, with the highest levels in the ovaries [87]. LPA_5 and LPA_6 expressions have not yet been reported for human tissue. The transcription factor PPARγ has been identified as an intracellular receptor for LPA [80], with activation of PPARγ by LPA essential for the formation of the neointima in atherosclerosis [107].

The number of unsaturated bonds and the position of the carbon chain (sn-1 or sn-2) on the glycerophosphate backbone significantly modulate the capacity of LPA to activate receptors [8]. LPA_3 has the most restricted ligand specificity, with the highest activity achieved when stimulated with LPA that contains unsaturated or polyunsaturated fatty acid chains with 18 carbons (C18:1, C18:2, C18:3), preferentially located on the sn-2 position. LPA_1 has the widest specificity. LPA_2 has low specificity for LPAs with short carbon chains (C12:0, C14:0) and is not affected by the sn-1 or sn-2 position of the fatty acid chain. Interestingly, most studies of LPA function and its receptors were carried out with the aid of 1-oleyl-LPA (C18:1).

LPA is water soluble but not active. It has maximum activity when bound to a carrier that interacts with LPA receptors. In vivo, LPA is primarily linked to albumin. Recently, the secreted form of gelsolin was shown to bind LPA with a higher affinity than does albumin (K_d gelsolin = 6 nM versus K_d albumin = 360 nM [41]). The fact that LPA binds to many carriers may have functional significance, including regulating bioavailability and protecting against degradation by PLs present in biological fluids [115]. Degradation may constitute a mechanism for cells to regulate LPA activity. Several members of the lipid-phosphate phosphatases (LPP) family that are expressed at the cell surface control LPA-dependent signal transduction pathways [13].

5.5 Expression of LPA Receptors in Skeletal Cells

Two 1955 reports [73, 123] were the first to demonstrate that osteoblastic cells responded to LPA and related lysophospholipids by MAP kinase activation to a rise in the cytosolic concentrations of ionized free calcium $[Ca^{2+}]_i$. Studies with RT-PCR revealed that bone and cartilage cells express four of the five known LPA receptors. Of these, LPA_1 is the most highly expressed receptor form, followed by LPA_2 and LPA_4 [21, 38, 75, 126]. LPA_3 transcripts are detected in osteoblastic cells, but are the least abundant of the LPA receptors. Mouse MLO-Y4 osteocytes express predominantly LPA_1 and LPA_2, with LPA_4 mRNA occurring only in trace levels [54]. As will be discussed below, the LPA_1 receptor is the functionally dominant form found in osteoblasts and osteocytes. Little is known about how osteoblasts respond to LPA, and even fewer studies have determined the effects of this lipid factor on chondrocytes. Kaplan et al. [53] first reported that sheep growth plate chondrocytes exhibited LPA-induced elevations of $[Ca^{2+}]$. These were pertussis toxin-sensitive, a condition consistent with a requirement for GPCRs. Kim et al. [62] subsequently determined that rat articular chondrocytes express LPA_1 and LPA_3, but not LPA_2. The mouse pre-chondrocyte cell line, ATDC5, expresses mRNA encoding LPA_1, LPA_2, LPA_3, and LPA_4 (Karin, unpublished data). RAW 264.7 macrophages, which can be driven into an osteoclast phenotype in vitro if

cultured in the presence of macrophage-colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) express mRNA encoding LPA_1 and LPA_2 (Karin, unpublished data). Additionally, mRNA encoding the receptors for LPA_1, LPA_2, and LPA_3 occur in mature osteoclasts that were generated in vitro from mouse spleen or bone marrow primary cells that had been treated with M-CSF and RANKL (Peyruchaud et al., in preparation). Recently, Heise et al. [45] synthesized VPC-12249, an antagonist of LPA_1 and LPA_3. When mouse bone marrow cells were treated with VPC-12249, the in vitro differentiation of multinucleated mature osteoclasts was inhibited (Peyruchaud et al. unpublished data). Osteoclasts in vitro may therefore also be subject to regulation by LPA.

In what follows, bone cell responses to LPA receptor occupancy will be categorized as either 'rapid' (seconds to minutes) or 'long-term' (hours to days). To be sure, some LPA actions, for example, cytoskeletal rearrangements, fit neither category. Yet categorizing LPA effects as fast or long-lasting is useful. Rapid responses can initiate signaling cascades that then induce long-term responses. As yet, however, in only a few instances have early signaling events been linked to long-term changes in bone cell physiology. Until more such instances are documented, we will have only limited understanding of the potential mechanisms by which LPA may regulate skeletal development and homeostasis.

5.6 Rapid Effects of LPA on Bone and Cartilage Cells

5.6.1 Ca^{2+} Signaling

Intracellular Ca^{2+} signaling regulates a wide variety of functional pathways in eukaryotic cells [9]. In osteoblasts it modulates many activities, usually in association with intracellular Ca^{2+}-binding proteins like calmodulin [135]. As expected when mediated by GPCRs, target cells like osteoblasts and chondrocytes respond to LPA exposure by acute elevation in Ca^{2+} [50, 53, 55, 61, 75, 79, 126]. LPA-induced Ca^{2+} transients in MC3T3-E1 cells were almost totally blocked when the cells had been pre-treated with pertussis toxin. This indicates a requirement for receptor linkage to $G_{i/o}$ proteins [75]. The mechanism by which ligand binding to GPCRs triggers Ca^{2+} signals involves the activation of PLC and the generation of the second messenger, inositol-1,4,5-trisphosphate (IP_3), which in turn activates ion release channels in the endoplasmic reticulum (ER). Consistent with this mode of action is that U73122, an inhibitor of PLC, prevents LPA-induced rise in $[Ca]_i$ in osteoblasts [126]. Cartilage cells also couple LPA receptors to IP_3-induced Ca^{2+} release from the ER. Likewise, the $[Ca^{2+}]_i$ elevations elicited by LPA in human articular chondrocytes are inhibited by prior treatment with thapsigargin which causes the intracellular Ca pool to become empty [28].

Treatment with the LPA receptor antagonist, Ki16425, greatly impairs the rise in intracellular $[Ca^{2+}]$. Ki16425 differs structurally from VPC-12249, and blocks LPA_1 and LPA_3 receptors to functions, and, at high concentrations, also blocks the LPA_2 receptor [79]. These data indicate that the primary mechanism by which LPA elicits Ca^{2+} signals in osteoblasts is stimulation of PLC activity that is associated with $G_{i/o}$ proteins coupled to LPA_1 receptors. The relative contribution to Ca^{2+} signaling of the other LPA receptors expressed by osteoblasts has not been determined. It is not known whether chondrocytes employ the same receptor coupling when LPA triggers elevations in $[Ca^{2+}]_i$. Whether LPA elicits Ca^{2+} signals in osteocytes or osteoclast has not been reported. Even though the mechanism by which LPA triggers Ca^{2+} signals is now known, the downstream targets are not known nor is the functional significance of this phenomenon understood.

5.6.2 MAP Kinase Activation

MAP kinase ERK is activated when osteoblasts are treated with LPA and is blocked by either PTH or phorbol ester [122]. This may involve the Raf-1 oncogene. LPA-triggered ERK phosphorylation in rat osteoblasts is not prevented by inhibition of phosphatidylinositol-3 kinase (PI3K) activity [38]. LPA stimulates the phosphorylation of the MAP kinases ERK and

p38 in rat chondrocytes within 5–10 min of exposure [57]. ERK is phosphorylated when human T/C-28a2 chondrocytes are treated with LPA [24]. Because ERK activation in rat chondrocytes was more significantly inhibited by pertussis toxin than was p38, the regulation of the two MAP kinases by LPA may be coupled to different G proteins.

Many instances exist in which the control of ERK activation has been linked to Ca^{2+} signaling [1]. Grey et al. [38] have reported that LPA-stimulated ERK phosphorylation in rat osteoblasts is blocked if extracellular Ca^{2+} is chelated with EGTA, but this is not the case if intracellular $[Ca^{2+}]_i$ elevations are eliminated by the cytosolic Ca^{2+} chelator BAPTA or by depleting ER Ca^{2+} stores with thapsigargin [38]. For this reason the role of osteoblast Ca^{2+} signals in LPA-enhanced ERK activation remains unclear.

The effects of LPA on target cells often involve the transactivation of EGF receptor-coupled signaling pathways [19]. Of the possible mechanisms responsible for this phenomenon, most of the evidence is in support of the LPA-induced cleavage of the protein ligands of the EGF receptor in the plasma membrane [90]. These findings and the report by Ahmed et al. [2] are consistent with a model in which transactivation of the EGF receptor is part of the signaling response. Yet PD 153035, another inhibitor of EGF receptor kinase activity, had little effect on ERK activation by LPA or on cell migration (discussed below, [55], unpublished data). Similarly, the AG1478 inhibitor has little impact on the ability of LPA to induce osteoblast differentiation [34]. It is therefore not yet clear how EGF receptor transactivation is involved in the responses of osteoblasts to LPA.

5.6.3 Cytoskeletal Rearrangements

A recently discovered rapid effect of LPA on primary rodent osteoblasts is the induction of bulbous membrane outgrowths, termed blebs, with a mean lifetime of 1.2 min [93]. Generating these structures must require rapid cytoskeletal rearrangements, because blebbing can be blocked by an inhibitor of Rho-associated coiled kinase (ROCK). Subsequent experiments revealed that the nucleotide ligands activated phospholipases D and A2, enzymes that are known to generate LPA from LPC and PA, respectively. Furthermore, chronic exposure to LPA desensitizes osteoblast receptors [74], and nucleotide-induced membrane blebbing did not occur in LPA-desensitized cells [93].

Other studies have linked the effects of LPA to rapid cytoskeletal changes in osteoblasts more directly. Cell–matrix and cell–cell attachments involve the coupling of the cytoskeleton to focal adhesion complexes. The activation (phosphorylation) of the focal adhesion kinases FAK and Pyk2 and the integrin-associated kinase p130 Cas was stimulated in MC3T3-E1 cells by both a 2- or 20-min treatment with LPA [15, 31]. Interestingly, the LPA-induced phosphorylation of Pyk2 was not blocked by pertussis toxin [31]. Both FAK and Pyk2 are involved in the formation of cell adhesion foci which are linked to the cytoskeleton. In their study of integrin receptor function, Carvalho et al. [14] took advantage of the property of LPA to rapidly (10 min) induce focal adhesion formation in chick calvarial osteoblasts. Even though the function of blebbing is not known, it appears to be a required step in the rearrangement of the cytoskeleton.

5.7 Long-Term Effects of LPA on Skeletal Cells

5.7.1 Cell Proliferation, Survival, and Differentiation

The maintenance of bone tissue, and the changes associated with osteoporosis, arthritis, and other skeletal pathologies, are closely tied to mechanisms that govern bone cell number [77, 130]. LPA is considered a growth factor because it stimulates mitogenesis and diminishes apoptosis [97] and may therefore play a role in the regulation of bone cell proliferation and survival. LPA stimulates [^{3}H]thymidine incorporation in MC3T3-E1 cells via a $G_{i/o}$ protein-coupled mechanism [15]. When primary human osteoblasts and osteoblast-like osteosarcoma cells were exposed to LPA for 10 min, DNA

synthesis was stimulated. This did not occur in pertussis toxin-treated cells. Variable effects on mitogenesis were observed after a 24-h LPA treatment [21]. LPA addition also protected primary rat osteoblasts from apoptosis due to serum deprivation [39]. Because LPA promotes osteoblast proliferation and survival, it may stimulate bone anabolism in vivo. If this is the case, LPA may have a therapeutic role in bone fracture healing. Whether LPA acts on osteoclasts is not currently known, but because of its anti-apoptotic effects on the closely related peritoneal macrophages [60], it may also act on osteoclasts.

ERK activity can be linked to multiple mitogenic and anti-apoptotic signaling pathways [81] and, interestingly, ERK activation or blockade of Src activity blunts the ability of LPA to stimulate osteoblast proliferation [15]. Cornish and colleagues [39] have reported that the mitogenic effects of LPA were not impaired by pharmacological inhibitors of ERK activation and that the anti-apoptotic effects of LPA on rat osteoblasts in vitro required $G_{i/o}$ proteins and PI3K activity, but were independent of ERK activation [39].

Whether LPA stimulates chondrocyte proliferation is not yet clear. In rat articular chondrocytes LPA stimulated [^{3}H]thymidine incorporation in a dose-dependent manner, a process that is inhibited by pertussis toxin or an inhibitor of ERK phosphorylation [57]. On the other hand, proliferation of T/C-28a2 cells, an immortalized line of human growth plate chondrocytes, was inhibited by 10 μM LPA [24]. Species differences or cell origin may be the reason for the discrepancy.

LPA may play a role in regulating 1,25$(OH)_2$ D_3-induced osteoblast differentiation [34]. Gidley et al. [38] reported that alkaline phosphatase, a marker of mature osteoblasts, was not elevated when the LPA receptor antagonist Ki16425 was added to a culture of human MG63 osteoblastic cells which contained 1,25$(OH)_2$ D_3. Lipid removal or albumin depletion had similar negative effects on alkaline phosphatase expression. The synergistic effect of 1,25$(OH)_2$ D_3 and serum on osteoblast maturation also was inhibited by pertussis toxin or an inhibitor of ERK activation. These findings indicate that LPA is a critical component for osteoblast differentiation.

5.7.2 Cytoskeletal Changes, Adhesion, and Migration

In addition to rapid changes in the cytoskeleton of osteoblasts, LPA, added to osteoblast cultures for 24 h, induced robust changes in F-actin geometry [34, 79], leading to alterations in stress fiber formation and to the appearance of long and thin membrane extensions. These morphological changes did not occur when the cells were treated with either pertussis toxin or Ki16425 [79]. Evidently osteoblast differentiation depends on the LPA-induced cytoskeletal rearrangements, inasmuch as the enhancement of alkaline phosphatase activity by LPA is blocked by an inhibitor of the F-actin regulator ROCK [34].

Cytoskeletal rearrangements are essential for cell movement to take place. Because LPA increases cell motility, it plays a role in endothelial cell migration during wound healing, angiogenesis [67], and cancer cell metastasis. LPA has been shown by both videomicroscopy and transwell migration assays to be a potent chemotactic stimulus for MC3T3-E1 pre-osteoblastic cells [79]. LPA-induced locomotion of pre-osteoblast was blocked by either pertussis toxin, Ki16425, or by blockade of PI3K activity with LY294002, consistent with a dominant role of the LPA receptor [52].

In the course of bone formation, osteoblasts, surrounded by mineralized matrix, differentiate into osteocytes, a post-mitotic cell with a highly dendritic phenotype [30]. These cells form an intercellular mechanosensory network in bone, the function of which is regulated by gap junctions that are formed at the dendrite termini [59]. Because LPA treatment of MC3T3-E1 osteoblastic cells leads to the formation of long, thin membrane extensions that resemble osteocyte dendrites [79], Karagiosis and Karin investigated the possibility that LPA would enhance osteocyte membrane outgrowth in vitro [54]. Dendrite outgrowth was found to be greatly enhanced by LPA in a dose-dependent manner, with maximal effect at 1.0 μM. Membrane extension was accompanied by an increase in F-actin stress fiber formation and was inhibited by pertussis toxin or Ki16425. Only LPA_1 and LPA_2 were detected in these cells; thus the effect

of Ki16425 could be attributed to inhibition of LPA_1 [54]. The assumption that dendrite outgrowth and cell motility may share a mechanism is supported by the ability of MLO-Y4 cells to exhibit LPA-induced chemotaxis, as well as dendrite outgrowth. Although LPA stimulates dendrite formation in osteocytes, it inhibits neurite extension [102]. These disparate findings highlight the cell-specific effects of LPA on its target cells.

5.7.3 Gene Expression

LPA has tropic effects on its target cells, but few reports describe how LPA regulates gene expression [35, 42, 66, 92, 112]. In a global gene expression analysis of LPA-treated MC3T3-E1 preosteoblastic cells, Waters et al. [126] found that over 500 transcripts were altered at least twofold. Gene ontology (GO) analysis revealed that many expression changes were linked to specific biological functions. As might be expected for a response to a mitogen, the largest number of LPA-regulated genes was associated with the GO category 'Cell proliferation'. LPA-treated MC3T3-E1 cells also displayed changes in gene expression in the GO categories 'Cell migration', 'Cell motility', 'Chemotaxis', and 'Organ development'.This may be relevant to the effects of LPA on osteoblast differentiation [34].

Perhaps the most novel result of the global expression study was the observation that LPA-treated osteoblastic cells also exhibit changes in 'Response to stress', a category that includes inflammatory modulators. Genes encoding proinflammatory cytokines were stimulated, but the anti-inflammatory gene products sST2, ST2L, and heat-shock protein 25 were among the most highly induced transcripts. Interestingly, LPA stimulation of these gene products appears to be governed by different receptor coupling mechanisms, inasmuch as pertussis toxin inhibited the LPA-induced expression of sST2, but not that of ST2L [126], even though they are spliced transcripts from the same gene [116]. These findings support the idea that osteoblasts play a role in regulating inflammation responses [78].

In the T/C-28a2 human chondrocyte cell line ornithine decarboxylase (ODC) activity increased 4–16 h after LPA exposure [24]. The response was sensitive to pertussis toxin and to inhibitors of Src, PI3K, and PKC-δ, but did not involve Ca^{2+} signals or ERK activity. The mechanism by which LPA regulates ODC activity was not known, but ODC transcription, translation, and/or protein degradation respond to a variety of growth factors [95]. This finding may therefore constitute the link between arthritic inflammation and the LPA effect on chondrocytes. Human MG63 osteoblastic cells exhibit elevated cyclooxygenase-2 (COX-2) protein expression after 6 h of LPA treatment [34], but whether this is the result of transcription or post-transcriptional events is not known.

5.8 Potential Regulatory Roles of LPA in Bone

Current understanding of how LPA acts on bone is limited almost entirely to findings from skeletal cell culture. Yet it seems reasonable to think this bioactive lipid regulates bone development and regeneration. Gene expression analysis of MC3T3-E1 cells has shown that LPA modulates the number of transcripts that encode proteins that modulate bone tissue development [126]. Deletion of LPA_1 expression led to mice with skeletal deformities, particularly in craniofacial bone [17]. The loss of LPA_1-coupled signaling in osteoblasts may be partly responsible for the defective bone development in the $LPA_1^{(-/-)}$ animals. LPA_1 receptor-mediated regulation may moreover be needed for proper formation of the growth plate cartilage which in turn is required for endochondral ossification.

5.8.1 Fracture Healing

A rarely addressed issue is where LPA originates and in what environment the cells encounter it. Degranulating platelets generate LPA during blood clot formation [22, 100], and preosteoblasts and osteoblasts are likely exposed

to high LPA levels (1–20 μM) in the vicinity of hematomas resulting from bone injury. RT-PCR analysis has demonstrated the presence of ATX transcripts in MC3T3-E1 pre-osteoblastic cells, in primary mouse calvarial osteoblasts, and in MLO-Y4 osteocytes (Karin, unpublished). Platelet-derived protein growth factors facilitate skeletal regeneration [114], but it is not known whether LPA and other lipid factors contribute to bone repair. Based on findings from culture studies it seems reasonable to think that the anabolic effects of LPA contribute to fracture healing.

Fracture healing involves four stages: (1) inflammation and hematoma formation; (2) chondrogenesis (soft callus formation) and neovascularization; (3) endochondral ossification; and (4) remodeling [23]. Much LPA constituting a rich local source is generated during blood clot formation in the fracture gap. LPA treatment of mouse osteoblastic cells leads to increased expression of pro- and anti-inflammatory genes [131]. In human osteoblast cultures LPA addition induced an increase in the pro-inflammatory enzyme COX [34]. LPA therefore plays a role in the regulation of osteoblast-mediated events that occur in the initial phase of fracture repair. Neovascularization is essential for proper bone formation and repair. Angiogenic factors such as VEGF foster skeletal regeneration [65, 110] and are modulated by the chemotactic effects of LPA on vascular endothelial cells [67]. It is also likely that platelet-derived LPA fosters new blood vessel formation in the course of fracture healing. New vasculature not only adds to the nutrient supply and waste removal at the fracture site, but also provides a conduit for chondrocyte migration and osteoblast progenitors. Because endothelial cells give rise to polymorphic cells that subsequently differentiate into osteoblasts [20], LPA-induced chemotaxis of endothelial cells may enhance bone formation and repair. The proliferation and migration of osteoblast progenitor cells to the fracture gap is essential for bone regeneration [23], and is modulated by LPA [15, 21, 38, 79]. LPA stimulation of osteocyte dendrite outgrowth may help in re-establishing the mechanosensory network in the newly synthesized bone [54].

5.8.2 Regulation of Mechanotransduction

Bone mass maintenance depends on physical forces and mechanical strain which induce interstitial fluid shear. This in turn regulates bone cell function via ion channels (especially Ca^{2+} permeability), cytoskeletal rearrangements, and altered gene expression [49, 99]. As described above, many interactions of LPA with osteoblastic cells mirror the effect of physical stimulation. LPA augments fluid shear-induced Ca^{2+} signaling in smooth muscle and endothelial cells [88], but the postulate that the effects of LPA on bone cells are synergistic with mechanical stimulation has not been tested. The LPA-induced osteoblast gene products sST2 and ST2L increased in mechanically strained cardiac tissue [128], but decreased in MC3T3-E1 cells exposed to fluid shear [126].

Physical stimulation also has a role in fracture repair in that new bone quality is improved if mechanical loading is applied to the damaged region during the early stages of healing [6, 37]. That is the time when cells in the fracture region encounter platelet-derived LPA. If osteocytes indeed constitute the major mechanosensors in bone [59], then osteocytes and chondrocytes in the fracture space would be exposed to LPA that diffuses into the interstitial fluid. LPA may therefore play a significant role in enhancing the response of osteoblasts and other mechanosensitive cells to physical stimuli in the course of early bone healing.

5.8.3 The Pathology of Arthritis

Inflammation is the net result of the interplay of pro- and anti-inflammatory cytokines, with pathology of arthritic cartilage due to system imbalance. Arthritis is associated with high serum levels of PLA2, an enzyme that can generate LPA from PA (Fig. 5.1) [84, 86, 96]. Although chondrocytes in culture are sensitive to LPA [24, 53, 57, 61], the in vivo effects of LPA on cartilage are not known. Chondrocytes multiply in response to LPA [57], and chondrocyte proliferation could conceivably counteract arthritis-linked apoptosis [64]. LPA treatment of

osteoblastic cells has led to increased expression of the anti-inflammatory gene products sST2 and ST2L [57], both of which have therefore been proposed as therapeutics for arthritis [70, 131]. On the other hand, LPA stimulates ODC activity [24], thereby causing an increase in pro-apoptotic polyamines, molecules that foster arthritis progression [28, 134].

5.8.4 Regulation of Bone Mass by Leptin

In 2000 Ducy et al. [23] reported that leptin, a peptide hormone associated with the control of body weight, also modulated bone mass homeostasis by inhibiting bone formation via a mechanism that involves the central nervous system. Eiras et al. [27] found that leptin inhibited LPA from inducing $[Ca^{2+}]_i$ elevations in a human embryonic kidney cell line and that this required PI3K and protein kinase C activity. Exposure of adipocytes to S1P, a bioactive lipid that is structurally and functionally related to LPA, led to inhibition in the insulin-stimulated secretion of leptin [52]. These findings point to a further pathway by which LPA may regulate bone formation.

5.9 LPA and Cancers That Metastasize to Bone

High LPA levels in ascites fluid and plasma from patients with ovarian and cervical cancers [13] may make it a marker for gynecological cancers. Quantitative RT-PCR analyses have shown that LPA_2 expression is high in colon cancers [106], inasmuch as LPA stimulates colon cancer cell proliferation and migration by activating LPA_2 and LPA_3 in a β-catenin-dependent signaling pathway [132]. Shida et al. [105] have suggested that the mitogenic activity of LPA is mediated through LPA_2 and LPA_3, whereas cell migration is driven by LPA_1. LPA_1 also regulates cell proliferation in breast and prostate cancer [11, 41]. Currently there is no information on the role of LPA and its cognate receptors on bone cancers.

5.9.1 LPA and Thyroid Cancer

Thyroid cancer cells express LPA receptors. LPA induces thyroid cancer cells to migrate and invade other tissues [56, 103]. Schulte et al. [109] have reported that lpa_2 gene expression, compared to that in normal thyroid and goiter, is elevated in both papillary and follicular cancer. Even though the overall expression of LPA_1 is unchanged in malignancy, increased expression of LPA_1 has been found to correlate with lymphonodular metastasis. These results suggest that evaluation of gene expression for LPA receptors might not yet have prognostic value.

5.9.2 LPA and Prostate Cancer

Prostate cancer cells express LPA receptors [18]. LPA_3 has been cloned from such cells because this receptor is highly expressed in this type of cancer [48]. LPA serves as a powerful mitogenic factor in prostate cancer cells [63], to the point that these cells were used to identify signaling pathways that were stimulated by LPA. Accordingly the mitogenic action of LPA involved transactivation of EGF and PDGF receptors [40, 125]. Prostate cancer cells produce LPA, which also acts as an autocrine mediator [129]. This may indicate the existence of an amplification loop for LPA action on prostate cancer cells. Remarkably, among genes of PC3 cells that are downregulated by genistein, an inhibitor of prostate cancer, lpa1 gene has the fastest and highest response [71]. Because the LPA receptor controls both migration and proliferation of prostate cancer cells [41, 43], LPA_1 may play a central role in the growth and progression of prostate cancers.

5.9.3 LPA and Breast Cancer

Recent reports have shown that primary breast cancers express LPA receptors, with LPA_2 expression higher than for LPA_1 and LPA_3 [58]. LPA induces the migration and proliferation of breast cancer cells and protects them against apoptosis [36]. We have shown that LPA also mediates the progression of bone metastases in breast cancer.

5.10 LPA and Bone Metastasis

LPA produced by activated blood platelets [5, 22] modulates local progression of bone metastases by stimulating tumor cell proliferation and the production of IL-6 and IL-8, two cytokines involved in osteoclast-mediated bone resorption [11]. Therefore, LPA-dependent cell signalling pathways may constitute new therapeutic targets for bone metastases. Apparently, only LPA_1 modulates progression of osteolytic lesions caused by hamster ovary CHO$\beta_3$wt cancer cells, inasmuch as these cells express only LPA_1 [11, 94]. Moreover, overexpression of LPA_1 in transfected human MDA-BO2 breast cancer cells led to a dramatic increase in the capacity of these cells to induce bone metastases in a nude mouse model [11]. Altogether these observations suggest that LPA_1 may play a major role in cancer-induced bone metastasis. To address this question we used genetic and pharmacological approaches in vitro and in vivo [12]. We found that LPA_1, in addition to its role in breast cancer cell proliferation, plays a significant role in bone destruction, as it stimulated production of IL-6, IL-8, GM-CSF, Groα, and MCP-1 cytokines involved in osteoclast functions. In ovarian cancer cells, LPA regulates IL-6 and IL-8 expression via a NF-κB pathway downstream of the activation of both LPA_{1-3} receptors [25]. NF-κB is a key mediator of LPA activity, because it regulates the LPA-induced survival of prostate PC-3 cancer cells that express LPA_{1-3} [98]. Activated NF-κB is associated with aberrant breast cancer cell proliferation and resistance to apoptosis [10]. It is therefore conceivable that the LPA-dependent production of cytokines and the proliferation of breast cancer cells are mediated through a LPA_1-NF-κB signaling pathway.

Using a small RNA interference strategy, we observed that silencing LPA_1 expression in breast and ovarian cancer cells markedly altered the progression of bone metastases, because tumor burden was reduced. This may make LPA_1 a target in the treatment of bone metastases. In order to antagonize LPA, drugs acting on LPA receptors have been developed. They include DGPP 8:0 [27], VPC-12249 [45], NAEPA [101], and Ki16425 [89], which, although structurally different, inhibit LPA_1-dependent and, to a lesser extent, LPA_3-dependent cell signaling pathways. No specific LPA_1 antagonist has been described so far. In our study, we demonstrated that Ki16425 mimicked the inhibitory effects of LPA_1, markedly impairing tumor cell proliferation and osteoclast recruitment. Pharmacokinetic analyses revealed that LPA_1 rather than LPA_3 was the main target.

To treat bone metastases, current treatment involves the use of bone resorption inhibitors to interrupt the cycle between bone resorption and tumor growth [85]. Because this treatment becomes ineffective with time, it is necessary to develop additional therapeutic strategies [46]. LPA_1 is expressed by tumor cells and may therefore play an essential role in bone metastasis progression. Targeting LPA_1 would thus become an effective treatment for bone metastases.

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6.

Role of Bone Microenvironment/Metastatic Niche in Cancer Progression

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6.1 Introduction

Much progress has been made in the detection and treatment of primary malignant tumors in recent years. However, the stages of advanced malignancy are less understood and fewer therapies have been developed specifically to target metastatic spread. Cancer metastasis remains the single most important cause of cancer-related deaths [83]. Tumor progression involves a cascade of distinct events, from local invasion, migration, dissemination in the circulatory system, and eventual establishment at organ-specific sites distant from the primary tumor. Much work has focused on the genetic mutations that confer a cell-intrinsic invasive and migratory phenotype, enabling dissemination and survival of tumor cells in the circulatory system. More recently, the importance of pre-conditioning of the external microenvironment of metastasizing cells by tumor-secreted systemic factors has been highlighted. Paget's theory first recognized the importance of a "congenial soil" in providing a receptive microenvironment for the disseminating cancer "seed" [61]. Over 100 years later, we have begun to identify the biological elements that contribute to the formation of a supportive site for the metastatic process. The blood–bone marrow axis is emerging as a key mediator in the transformation from a primary tumor to widespread metastatic disease. Tumor-secreted cytokines, chemokines, and disseminated tumor cells themselves home to the bone marrow, effecting changes in the bone marrow stroma and stem cell niches. Conversely, bone marrow-derived factors and cells of the hematopoietic system migrate to the periphery to prime distant sites for the arrival of tumor cells and to establish a favorable microenvironment for tumor progression, the metastatic niche. This chapter focuses on recent advances in the understanding of the role that bone marrow-derived cells and bone marrow stroma play in cancer progression and metastasis. Parallels will be drawn between stem cell niche dynamics within bone marrow and pathological niches for benign and malignant cells at metastatic sites. Potential pharmacological and therapeutic targets in this setting also will be discussed.

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6.2 Tumor-Derived Growth Factors, Cytokines, and Chemokines Mediate Early Changes in the Bone and Tumor Microenvironment

The dialog between the primary tumor, its microenvironment, and the bone is mediated by tumor-secreted growth factors, cytokines and chemokines. A variety of these factors are secreted by the primary tumor and surrounding stroma throughout the metastatic progression, modifying the primary tumor microenvironment, priming distant sites in the bone and mobilizing bone marrow-derived cells. The following section will highlight the cytokines and growth factors that are integral to the malignant process.

6.2.1 Angiogenic Growth Factors – The VEGF Family Proteins

Tumor-derived factors may act in an autocrine, paracrine, or systemic fashion to orchestrate activation of locally embedded cells and the recruitment of mature cells and progenitor cells of the immune and hematological system, thus creating a microenvironment conducive to malignant progression. Vascular endothelial growth factor (VEGF) is perhaps the best-characterized cancer-associated growth factor. It is expressed by nearly every tumor type examined [18] and has many diverse roles in the malignant process. It is therefore a highly attractive pharmacological target. The VEGF receptors VEGFR1 (Flt-1), VEGFR2 (Flk-1), and VEGFR3 (Flt-4) mediate complementary and synergistic effects in the promotion of malignant progression, orchestrating new vessel formation and vascular maintenance [18]. Within the bone marrow, VEGF has important autocrine and paracrine effects on hematopoietic cell growth and bone formation, promoting osteoblastic differentiation and direct expansion of the hematopoietic progenitor cell (HPC) population [24, 30, 65, 77]. Autonomous secretion of VEGF by tumor cell lines may have autocrine effects to promote cell survival, proliferation, and motility [84]. In addition, VEGF has important paracrine and systemic effects, and is one of the most potent angiogenic factors known.

Tumor hypoxia induces expression of the VEGF family proteins via hypoxia-inducible factor (HIF). This, in turn, induces the mobilization and recruitment of VEGF-receptor expressing cells. Formation of tumor neovasculature requires incorporation of locally embedded endothelial precursors as well as "hemangiogenic" cells recruited from the bone marrow [46]. Bone marrow-derived endothelial progenitor cells (EPCs) express VEGF receptor (VEGFR-2) and share a common lineage with HPCs, expressing VEGFR1. These cell types are recruited from the bone marrow to malignant tissues, and both are required for stable and organized vasculogenesis. $VEGFR2^+$ cells directly participate in the formation of new endothelial cells and $VEGFR1^+$ cells can be seen perivascularly; this suggests that these cells confer vessel stability and promote tumor progression. Mice with a deficient inhibitor of the differentiation (Id) gene have defective angiogenesis due to impaired mobilization of both EPCs and HPCs from the bone marrow. They are also tumor resistant. Transplantation of wild-type bone marrow in these mice restores wild-type tumor angiogenesis and normal tumor growth [46].

Placental growth factor (PlGF) is a distinct member of the VEGF family. Unlike VEGF-A, which may bind to any of the VEGF receptors, PlGF signals only via VEGFR1 [67]. PlGF is important during embryogenesis and pregnancy. Unlike VEGF it is expressed in adulthood only in pathological conditions. Tumors that co-express both PlGF and VEGF tend to have a more malignant and invasive phenotype [41, 47, 82]. This highlights the importance of VEGFR1 over the other VEGF receptors in cancer metastasis. Many leukemia cells, including acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and acute lymphoblastic leukemia (ALL), produce high levels of VEGF and express VEGF receptors [42, 65]. In liquid tumors VEGF orchestrates tumor progression and VEGFR1 determines localization of leukemic cells within the mar-

row and regulates their survival and exit into the circulation [20].

6.2.2 Other Growth Factors

In addition to the angiogenic growth factors VEGF and PlGF, fibroblast growth factor 2 (FGF-2) is also abundantly expressed by many tumor types. By activating locally embedded fibroblast-like cells at the metastatic site and in the bone marrow, FGF-2 may play an important role in metastasis. Activation of these cells leads to up-regulation of fibronectin expression and of other extracellular matrix proteins within the microenvironment of the primary tumor, in the marrow, and at distant metastatic sites. In the bone marrow, fibroblasts and fibronectin contribute to the architecture of the niche of the hematopoietic stem cells (HSCs) and to regulation of cell cycling [5]. Fibronectin also provides a "scaffold" or adherence platform for incoming tumor cells that are disseminated by the primary tumor.

Platelet-derived growth factor (PDGF) also plays a pivotal role in cancer metastasis [78]. PDGF is responsible for enhanced expression of extracellular matrix adhesion proteins such as hyaluronic acid, proteoglycan, fibronectin, and collagen. In addition, it induces the recruitment of pericytes to stabilize newly forming tumor blood vessels [1, 22]. Furthermore, PDGF is also implicated in the selective metastasis of prostate and breast cancers to bone, with PDGF mediating interaction with the bone marrow stroma that confers a survival advantage in this new microenvironment [87]. Transactivation of the PDGF receptor alpha by bone marrow stromal cells activates the Akt pathway [14].

6.2.3 Pharmacological Inhibition of the Soluble Factors that Promote Metastasis

Inhibiting cancer-associated growth factors may slow metastatic progression. Therapeutic approaches that block VEGF have significantly slowed progression and improved survival in certain advanced cancers [19]. Bevacizumab, an anti-VEGF monoclonal antibody, was approved by the US Food and Drug Administration (FDA) in 2004 for the treatment of metastatic colorectal cancer. It is under trial in other malignancies, including lung and kidney. Several other inhibitors of the VEGF signaling pathway are currently undergoing clinical trials. They include antibody-based and small-molecule inhibitors such as SU11248 (sunitinib) and Bay 43–9006 (sorafenib), receptor tyrosine kinase (RTK) inhibitors that disrupt VEGFR signal transduction [19, 69]. STI571 (Gleevec, Imatinib Mesylate), a protein kinase inhibitor licensed for the treatment of CML and kit^+ gastrointestinal stromal tumors, also inhibits the PDGF receptor and c-Kit (SCF). It also reduces metastasis of pancreatic carcinoma in animal models [29].

Efforts to block growth factors and chemokines in malignancy are complicated by the complexity of these pathways and the natural redundancy of the relevant growth factors and receptors. Notwithstanding the fundamental role of VEGF in tumor angiogenesis, new blood vessels may develop even if VEGF is blocked, possibly because resistance to therapy has developed. A reduction in VEGF may be paralleled by an increase in other growth factors involved in metastatic spread, such as PlGF [10]. Also, vessel density may be somewhat independent of vessel function. The Delta-like ligand 4 (Dll4) is important in the genetic regulation of angiogenesis via the Notch pathway. When this ligand is blocked, angiogenic sprouting increases, as does branching and vessel density, but vessel function decreases paradoxically [58]. This illustrates the importance of thoroughly understanding the molecular mechanisms. Because VEGFR1-expressing cells stabilize new tumor vasculature, targeting VEGFR1 and Dll4 may constitute a novel and effective therapy.

6.3 Within the Bone Niche–Niche Interactions Promote Tumor Progression

Throughout ontogeny and in adult life HSCs circulate and migrate in a highly specific fashion to the niches in the bone marrow and to regenerative sites in the periphery. A dense mesenchymal

cell-derived stromal cell network and extracellular protein scaffold set up a highly organized microenvironment that supports HSC proliferation and differentiation in the bone marrow [44]. At the marrow periphery, HSCs are closely associated with osteoblasts in the endosteal niche where they maintain a primitive and quiescent state [57, 73]. A sinusoidal vascular network traverses the marrow and harbors the vascular niche, supporting HSC proliferation, differentiation into HPCs, and transendothelial migration of the more differentiated daughter cells [31]. The physiological and molecular scaffold that supports HSC proliferation and maintenance in an undifferentiated state can be greatly amplified in times of physiological stress, as situations of trauma wounds or in advanced malignancy. Disseminated cancer cells use some of the same signaling machinery to survive in the circulation, to "home" to specific secondary sites, and to proliferate in modified bone and metastatic niches.

Tumor cells have been found in the bone marrow of patients with malignancy, even if distant metastases are not overt. Although the clinical significance of these cells is not fully understood, the bone marrow seems to provide a microenvironment that protects tumor cells from chemotherapeutic agents. These tumor cells tend to remain in a dormant and quiescent state until reactivated. The interaction between malignant cells and the bone microenvironment also may serve as a "training ground", providing molecular education and altering gene expression of cells to effect a more aggressive phenotype.

6.3.1 Stem Cell and Tumor Cell Migratory Pathways

Migration and homing mechanisms of stem cells are paralleled in metastatic disease, with some chemokines implicated in the pairing of tumor cells to specific metastatic targets. Arguably the most important chemokine that governs migration patterns of hematopoietic cells throughout embryogenesis and adult life is stromal-derived factor-1 alpha (SDF-1α, also known as CXCL12). SDF-1α is a key player in cancer biology and metastasis, and interactions among SDF-1α and its receptors, CXCR4 and CXCR7, are important for tumor progression [9]. High concentrations of SDF-1α are needed for hematopoietic cells to be able to home to their endosteal niche in the bone marrow. SDF-1α also mediates the homing to bone of CXCR4-expressing tumor cells.

In addition to secreting large amounts of SDF-1α, osteoblasts also express anchorage molecules that include angiopoietin (Ang-1) and osteopontin (Opn). Adhesion to these factors not only retains stem cells within the niche, but also governs their replication kinetics and quiescence. Recently, data have linked Opn with metastasis of breast, colon, prostate, and liver tumors [34, 81].

In theory, inhibitors of the CXCR4–SDF-1 axis could be utilized against metastasis. Given however, SDF-1α's key role in stem cell signaling, any clinically useful therapy must selectively target pathological SDF-1α/CXCR4 signaling in malignant cells, yet preserve homeostatic stem cell functioning. Strategies using antisense oligodeoxynucleotides or selective RNA-mediated interference (siRNA) have potential for selectively targeting CXCR4 expression in malignant cells [40].

6.4 Bone Marrow-Derived Cells Are Recruited to Primary Tumor, Promoting Invasion, Migration, and Dissemination

Tumor-secreted factors mediate key changes in the tumor and bone microenvironments. This includes matrix remodeling, alterations in vascular permeability and neovascularization, and promoting the progression from an in situ carcinoma to an invasive and disseminating lesion. These changes occur as tumor cells acquire migratory properties, including loose adherence to stroma and evasion of anoikis [49]. Breakdown of the extracellular matrix and tumor cell invasion require matrix metalloproteinase (MMP) expression. Cleavage of other cytoactive factors is also important for metastatic progression [21, 60]. Altered cellular expression of adhe-

sion factors permits cells to detach from the primary tumor and traverse the parenchyma to extravasate into the circulation [23].

The development of a well-vascularized stroma is essential for growth of the primary tumor, for invasion of tumor cells into the circulation, and for progression of micrometastases to metastatic disease. True "angiogenesis" means that vessels are formed by sprouting from existing mature vasculature. Within tumors, vessels develop by "vasculogenic" processes. At the primary tumor site, contrary to previous understanding, vasculogenesis occurs early and is not entirely a function of tumor size. $VEGFR1^+$ HPCs and $VEGFR2^+$ EPCs are recruited to the tumor site earlier in the malignant process than can be explained by tumor expansion alone. Tumor types differ in their continued dependence on the $VEGFR1^+$ HPCs and $VEGFR2^+$ EPCs for vasculogenesis.

The term "epithelial–mesenchymal transition" (EMT), describes the change that tightly adherent epithelial cells undergo during embryogenesis to become highly mobile mesenchymal or neural crest cells [86]. The term also applies to the loss of adherence to the primary tumor and to local invasion and migration. These changes are the result of upregulation of several EMT-inducing genes within the primary tumor, including Snail, Slug, and Twist, and also of signaling via the transforming growth factor-beta (TGF-β) receptor [8, 48]. TGF-β has biphasic effects during tumorigenesis. Early in tumor expansion, TGF-β is an inhibitor of tumor growth. During later stages, perturbation of the TGF-β signaling pathway and autonomous TGF-β production by tumor cells may confer an invasive and aggressive phenotype, promoting metastatic progression [3]. Therefore, therapeutic approaches should aim to promote the TGF-β-induced growth inhibition and apoptosis, but inhibit the signaling pathway that mediates cellular invasion and metastasis. Further understanding of the TGF-β signaling pathway may lead to the development of small drug molecules that inhibit specific molecular steps and inhibit the deleterious actions of the pathway.

Tumor cells at the "invasive front", or peripheral tumor edges, are exposed to the surrounding tissue parenchyma and communicate with immune cells, resident fibroblasts, and macrophages [12]. These interactions enhance selection of cells that can survive without adhering to neighboring cells and that are more hypoxia-resistant. These disseminated cells are also more likely to survive in distant tissue sites where activated fibroblasts, immune cells, and hematopoietic cells mimic the invasive edge of the primary tumor and reconstitute a parallel environment. Expression of lysyl oxidase (LOX), an extracellular matrix enzyme, is stimulated by tumor hypoxia. Hypoxia in turn induces stromal invasion by primary tumor cells, while simultaneously promoting cell–matrix adhesion at distant sites to which the tumor cells will ultimately metastasize [16]. LOX is particularly interesting because it has both intracellular and extracellular functions. LOX was initially identified as a tumor suppressor, but we now know that its expression is both reduced and elevated in human cancers. Therefore, anti-LOX strategy must promote metastatic progression, yet spare other cellular functions. Antibody inhibition of LOX has been effective in decreasing metastasis and prolonging survival in animal studies [17].

6.5 Accessory Bone Marrow-Derived Cells Support Tumor Cell Survival and Dissemination

Mature cells of the immune and hemostatic systems have long been known to be also involved in carcinogenesis and metastasis. This includes macrophages, lymphocytes, and platelets. Inhibiting cytokines produced by inflammatory cells have excellent efficacy in reducing metastasis. For example, when the macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine overexpressed by prostate cancer cells, was inhibited, tumor progression was slowed [51, 52]. Furthermore, as is also true for bone marrow-derived cells, mature immune cells can be used to deliver specific anti-inflammatory or anti-tumor agents.

Other hematological cells, including platelets, also may play a role in tumor metastasis. The first event in tumor cell invasion at distant sites is lodgment and adhesion at the local vasculature. Formation of platelet microthrombi may be part of this process. Furthermore, platelets may act as a "shield" to prevent immune attack by encompassing disseminated tumor cells while in the circulation.

More recently, platelets have emerged as key players in directing homing and providing retention signals for bone marrow-derived and tumor cells. Platelet-deployed SDF-1α is critical for recruitment and retention of $CXCR4^+$ HPCs and EPCs in revascularization of ischemic tissue and at sites of tumor angiogenesis [31]. Local activation and release of SDF-1α by platelets also may govern migration patterns of $CXCR4^+$ tumor cell lines. Furthermore, megakaryocytes and platelets are major producers and storage vehicles for both pro- and anti-angiogenic factors. The number of megakaryocytes and platelets, and their content of thrombospondin, a very potent natural anti-angiogenic factor, is a determinant of the angiogenic phenotype [39]. What determines that these cells produce and release pro- or anti-angiogenic factors is not known, but making use of this mechanism may provide a novel therapeutic approach to tumor angiogenesis and may overcome the pathological functioning of bone marrow-derived cells in malignancy.

6.6 Bone Marrow-Derived Cells Are Recruited to Sites of Future Metastasis, Forming the "Pre-metastatic Niche"

Tumor-derived factors, including VEGF-A, TNFα, and TGF-β, have been shown to mediate preparatory changes within the organs of future metastasis in advance of tumor cell arrival – in other words, much sooner than previously recognized [35]. The changes involved include matrix remodeling, stromal differentiation, and recruitment of bone marrow-derived cells. The altered tissue parenchyma at distant sites from the primary tumor facilitates metastatic progression. It is termed the pre-metastatic niche.

6.6.1 Early Stromal Changes at Sites of Future Metastasis

Cells from the bone marrow that form clusters within tissues at pre-metastatic sites recapitulate features of physiological stem cell niches. Fibroblasts and fibroblast-like cells, resident at the pre-metastatic sites when activated, upregulate fibronectin synthesis. In turn bone marrow-derived cells preferentially adhere to niche structures via cell surface receptors with the aid of fibronectin [35, 63]. Similarly, the "pockets" of fibronectin expression at the pre-metastatic sites may determine where the migrating bone marrow-derived cells localize, giving rise to a metastatic pattern within an organ.

6.6.2 Tumor-Secreted Factors Preparing Distant Sites for Future Metastasis

The inflammatory chemoattractants S100A8 and S100A9 are induced in the lung prior to the arrival of disseminating tumor cells [27, 64]. Neutralization of these soluble factors diminishes expression of S100A8 and S100A9, and reduces migration of Lewis lung carcinoma (LLC) cells into pre-metastatic sites. These findings confirm and extend original observations by Kaplan et al. [35], which documented clusters of bone marrow-derived VEGFR1-expressing HPCs in sites of future metastasis and defined the pre-metastatic niche. In addition to a role in neovasculogenesis within the primary tumor, $VEGFR1^+$ HPCs are essential for preparing metastatic sites. When mice receive immunofluorescent-labeled bone marrow by transplantation, bone marrow-derived HPCs form clusters in distant organs and thus outline sites of metastatic invasion prior to the arrival of tumor cells. The location of the pre-metastatic clusters reflects the unique patterns of metastatic spread that characterize individual tumor types. For instance, in mice inoculated with B16 melanoma, which tends to metastasize widely to all organs, pre-metastatic clusters occur in the lungs, liver, testes, spleen, and kidney. In contrast, mice injected

with LLC develop clusters in the lung only. Moreover, administration of media conditioned by melanoma cells reprograms the metastatic profile of lung carcinoma to the profile of melanoma.

The expression of a unique cytokine profile may in part explain the distinct metastatic patterning observed with specific tumor types. While both LLC and B16 melanoma cell lines secrete high levels of VEGF, a high level of PlGF was found only in the melanoma-conditioned media. This highlights the integral role of VEGFR1 signaling in metastasis.

6.6.3 Stem Cell Niche Dynamics at Pre-metastatic Sites

Cell–cell and cell–matrix interactions at the pre-metastatic sites induce MMP expression, degrading the basement membrane, and permit tumor cell invasion into the tissue parenchyma. The VEGFR1$^+$ HPC clusters adhere via the surface receptors VLA-4$^+$, communicating with local tissue and establishing a true pre-metastatic niche. In the niche Id3 is expressed, MMP-9 is upregulated, integrin is activated, and SDF-1 is produced. All this attracts circulating tumor cells. Id genes are critical for organogenesis during ontogeny, but in the adult are predominantly expressed in pathological processes, including malignancy. Id gene expression is involved in the mobilization of cells from the bone marrow to angiogenic sites, in stromal cell interactions, and integrin expression [66]. The Id gene may therefore constitute a selective therapeutic target. The genetic changes associated with the metastatic microenvironment are only now beginning to be identified. Integrin expression such as VLA-4 expressed on the HPC and some tumor cells can bind to VCAM on inflamed endothelium, and to fibronectin within tissue parenchyma. Id proteins, DNA helix-loop-helix transcriptional regulators interact and modulate cellular proliferation and differentiation. These genes become activated in the bone marrow-derived cells in response to tumor-conditioned media. They are also active in tumor cells [33].

6.6.4 Pre-metastatic Lymph Nodes

When a tumor spreads to sentinel lymph nodes, this is a sign of poor prognosis and of the metastatic propensity inherent in most solid cancers. Even tumors that tend to metastasize primarily via blood vessels show dissemination to local lymph nodes. As histopathological techniques advance, the true definition of a "positive" lymph node becomes more complicated, as current immunohistochemical techniques can detect even a single migrating tumor cell [55]. With the aid of a transgenic spontaneous lymphoma model, it has been shown that VEGFR1$^+$ HPC clusters occur prior to the onset of lymphogenesis [35]. In humans VEGFR1$^+$ clusters are only found in breast carcinoma, but not in benign disease. In the future, identification of bone marrow-derived cells or fibronectin upregulation may become part of risk stratification and, when observed, may indicate a need for therapeutic intervention so as to inhibit or at least delay the onset of cellular metastasis.

Similarly, increased expression of specific growth factors by malignant cells may be an important indicator of lymphatic metastasis. Lymphangiogenesis within the primary tumor is a marker of a more advanced tumor stage. Expression of VEGF-C and VEGF-D by tumor cells has been implicated as indicating lymphangiogenesis and directly contributing to cancer metastasis [26, 68, 70]. High levels of VEGF-D and its receptors VEGFR2 and VEGFR3 are associated with advanced-stage metastatic prostate cancer [36].

6.6.5 Therapeutic Inhibition of the Pre-metastatic Niche

VEGFR1$^+$ cells appear to contribute functionally to the development of metastasis, because antibody blocking of VEGFR1 in an experimental murine model inhibited metastasis [35]. These cells and their molecular signaling pathways may therefore prove to be suitable targets for anti-metastatic therapies. Further characterization of these VEGFR1$^+$ HPCs will suggest potential pharmacological targets.

The recognition that microenvironmental changes occur in distant tissues before they are invaded by metastasizing cells has opened new avenues for the design of therapy that inhibits the early stages in the metastatic process. Some insight into how these niches

function in malignant disease may come from analyzing the physiological pathways of bone marrow-derived stem/progenitor cells. In particular, the requirement of stem cells for specific microenvironments, or "niches," may lead to the design of anti-metastatic agents and the prevention of a malignant microenvironment. The concept of "niches" was first coined to describe the highly defined and specialized structural scaffolds of the bone marrow in which hematopoietic cells reside [2]. Here, interactions between stem cell and niche components govern the kinetics of stem cell quiescence, self-renewal, proliferation, and differentiation. The result is a dynamic and responsive reservoir that supports hematopoiesis throughout adult life [25].

Another strategy for targeting metastatic disease would be to manipulate migrating bone marrow-derived cells themselves, rather than their signaling pathways. The bone marrow-derived cells that home to the tumor neovasculature and pre-metastatic sites could be used as "magic bullets", delivery vehicles for anti-cancer strategies [4]. The feasibility of integrating a suicide gene into bone marrow-derived progenitors to reduce tumor size and vascularity has already been confirmed in several animal studies [38, 45]. Not only HPCs and EPCs but also mesenchymal stem cells (MSCs) migrate to tumor sites, and MSCs may prove particularly useful as carriers of oncolytic adenoviruses, because they are only semi-permissive for viral replication. This limits viral lytic activity so that infected cells survive to reach the target site. A particularly vivid in vivo portrayal using bioluminescent imaging has shown that luciferase-labeled, mesenchymal progenitor cells which are infected with oncolytic adenovirus home directly to ovarian-tumor cells [32].

6.7 Metastatic Cancer Stem Cells Home Back to Bone

Non-malignant bone marrow-derived stem/progenitor cells contribute to malignant progression, and cancer cells may themselves possess stem cell-like properties. The concept of cancer stem cells (CSCs) is particularly important for the design of therapeutic agents. If CSCs, like physiological stem cells, remain quiescent and divide infrequently, they may evade traditional anti-mitotic chemotherapy or radiotherapy targeted to cells that have a high turnover rate. For example, CSCs in glioma are resistant to radiotherapy because they have an increased capacity for DNA repair [6]. The origin of CSCs is highly controversial. According to one hypothesis, physiological stem cells mutate to acquire a malignant phenotype. Other hypotheses suggest that a malignant cell that inhabits a physiological stem cell niche may acquire stem cell-like properties. Indeed, CSCs may be responsible for disease relapse, and like HSCs provide a reservoir of malignant cells by differentiation, while at the same time replicating and thus maintaining the cancer stem cell pool.

Whatever their origin, CSCs are critical for metastasis. One therapeutic approach would be to impede or to induce cellular differentiation. This has been achieved with all-*trans*-retinoic acid in the treatment of hematological malignancy because all-*trans*-retinoic acid induces differentiation of leukemic stem cells in acute pre-myelocytic leukemia [37]. An alternative approach is to target the homing mechanisms by which CSCs migrate to metastatic sites. Another approach is to try inhibiting the factors that govern the balance between CSC quiescence and active proliferation. As CSCs in solid tumors are characterized further, other potential therapeutic targets may become apparent that may inhibit CSCs in metastatic sites.

The "hijacking" of physiological stem cell mechanisms by CSCs constitutes a problem for therapy design, because the therapy must target the various CSC pathways, yet not in any way affect physiological stem cell functioning. Targeting the "pathological niche", i.e., the dysregulated microenvironment that supports the CSC, may be one approach. It is clear that niche occupancy confers a survival advantage to the metastasizing cells. Therefore both CSC and CSC-niche must be targeted simultaneously for successful anti-metastatic therapy.

6.8 Genetic Regulation of Metastasis

Because the propensity for metastasis is determined early in the cascade of events that lead to tumorigenesis [7, 28, 80], it has been of interest to study their genetic basis. Gene expression "signatures" have been correlated with overall tumor metastatic efficiency [79] and genes, the expression of which predicts metastasis to a particular organ, have been identified, as have profiles for bone- and lung-specific tropism of subpopulations of breast cancer cells [34, 53, 54]. Specific expression profiles, of surface receptors such as vascular cell adhesion marker (VCAM), MMPs, and chemokines (CXCL1), as well as of Id genes, enable a tumor cell to adapt and survive in a new microenvironment. These signatures are organ-specific, create a survival advantage and enable proliferation at distant sites. Targeting these genes along with those that give rise to the microenvironment may be a useful approach to inhibiting tumor spread.

6.8.1 Tumor Suppressor Genes

The mutation of tumor suppressor genes (TSGs) is clearly essential for tumor proliferation and its growth. TSGs may also regulate the expression of factors involved in tumor cell dissemination. An important transcriptional regulator of cell cycling is the p53 protein. Deactivating mutations of p53 inhibit its ability to recognize and repair DNA damage. They also prevent cellular apoptosis, thus permitting dysregulated cells to survive and proliferate [76]. The mutated p53 pathway has also been shown to be involved in tumor cell migration and in angiogenesis [74]. For example, p53 downregulates expression of the chemokine receptor CXCR4 that mediates stem cell homing to the bone marrow [50]. Loss of p53, and also of the von Hippel–Lindau TSG, promotes CXCR4 expression by breast cancer cells and their selective migration to distant organs where stromal-derived factor-1, its only chemokine ligand, is highly expressed [50]. High expression levels of the chemokine receptors, CXCR4 and CCR7, by human breast cancer cells are associated with increased chemotaxis, migration, and invasion of the target organs in which, in turn, the respective ligands SDF-1α and 6Ckine are highly expressed [56]. Similarly, KLF6 is a TSG that is commonly inactivated in a wide range of human cancers, including prostate, ovarian, and colorectal carcinomas. Originally identified as a regulator of cellular proliferation and apoptosis, KLF6 also modulates the expression of E-cadherin [13]. The "switch" from E-cadherin expression, mediating tumor cell adherence, to N-cadherin in mesenchymal cells enables stromal invasion, a critical step that makes possible metastasis and organ invasion [11].

6.8.2 Metastasis Suppressor Genes

Not all tumor cells metastasize. Cells must be able to extravasate and survive in the circulation, before reaching a site they invade and where they replicate. Given these requirements, it is not surprising that a class of genes exists that regulates metastasis (as opposed to tumorigenesis). Loss of the "metastasis suppressor genes" (MSGs) enhances metastasis without affecting primary tumor growth [15, 71, 72]. Twelve MSGs have been identified so far. Mutations of these genes enable tumor cells to acquire the ability to survive in a "foreign" microenvironment at distant sites. This is when apoptotic evasion, cell–cell and cell–matrix interactions, and adherence and stromal invasion by surface ligand–receptor pairing and protease secretion take place. KiSS-1 (also known as metastin) is an example of MSG. It is found in melanoma and breast cancer cells that have metastasized where it regulates the expression of MMP-9 [85]. Another MSG, KAI1/CD82, associated with many malignancies, including lung and prostate cancer, is thought to inhibit tumor cell invasiveness and motility via its interactions with surface integrins and growth factors, without affecting growth of the primary tumor [43].

6.8.3 Genetic Targeting

By targeting tumor cell-specific genes it may become possible to reduce primary tumor

growth, as well as tumor cell dissemination to distant sites. Pre-clinical studies indicate that application of p53-rescue drugs PRIMA-1 and CP-31398 decreases the expression of CXCR4 in breast cancer cell lines and reduces their invasive capacity [50]. Whether these drugs are effective in inhibiting tumor metastasis in vivo remains to be demonstrated. Therapeutic enhancement of MSGs also shows promise. Nm23, the first MSG to be identified, regulates the ERK-MAPK receptor signaling pathway and cell adhesion. Although mutations of Nm23 are rare, dexamethasone and medroxyprogesterone acetate therapy may enhance its expression. In experimental animal models of metastatic breast carcinoma, administration of these drugs led to reduced colonization of the lung by disseminating breast cancer cells [59, 62]. Where enhancement of MSGs is not possible, therapy was attempted by modulating downstream signaling pathways [75].

Gene expression profiling provides valuable information regarding cell-specific genetic metastatic propensity, but may not be the only approach. Microarrays may provide information on the genomic and proteomic alterations in the cells of the malignant microenvironment, thus shedding light on the molecular and cellular phenotype of the stromal cells.

6.9 Therapeutic Implications

Scientific advances in the understanding of metastasis are opening up a new period of cancer therapeutics. Anti-metastatic therapy requires targeting the metastasizing cells and their supportive microenvironment, and, because changes in the pre-metastatic microenvironment occur very early in tumorigenesis, anti-metastatic agents must become part of the initial primary tumor therapy. Genetic profiling of the primary tumor may identify those patients who would most benefit from early treatment with anti-metastatic agents. The complexity of the metastatic process demands a multi-modal approach to avoid therapeutic resistance and mutational evasion. Ameliorating the burden of morbidity and mortality of metastatic disease remains challenging, but new insights into the mechanisms of metastasis are likely to yield novel therapeutic approaches.

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7.

Detection and Characterization of Disseminated Tumor Cells present in Bone Marrow of Cancer Patients

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7.1 Summary

Early tumor cell dissemination occurs even in patients with small solid tumors, and bone marrow (BM) is a common homing organ for blood-borne disseminated tumor cells (DTCs) derived from primary tumors. Immunocytochemical or molecular assays allow the detection of a single DTC in BM at a frequency of one tumor cell in one million surrounding hematopoietic cells; e.g., tumor cells are frequently detected in the BM of breast cancer patients without clinical or even histopathologic signs of metastasis. Evidence has emerged that the detection of DTCs and circulating tumor cells (CTCs) in blood may provide important prognostic information and, in addition, might help to monitor efficacy of therapy.

The characterization of DTCs has shed new light on the complex process underlying early tumor cell dissemination and metastatic progression in cancer patients. Characterization of DTCs should help identify novel targets for biological therapies aimed at preventing metastatic relapse and to monitor the efficacy of these therapies. It is crucial, however, to improve and standardize methods for the detection of DTCs. In this chapter, we summarize the clinical background and the technical aspects of current methods used for the detection and characterization of DTCs in BM.

7.2 Background: Clinical Relevance of DTC Detection in BM

Notwithstanding the progress achieved in treatment of solid tumors such as breast cancer, the prognosis of patients even with small primary tumors is still limited by metastatic relapse that often occurs a long time after removal of the primary tumor. The relapse thus is an indication of an early systemic spread of the tumor cells. The development of rare cell detection techniques has made possible the demonstration in large patient cohorts that DTCs in BM are detectable in 20–40% of breast cancer patients, even though they have no overt signs of distant metastases [23]. Most available data are for breast cancer, but similar prevalence of DTCs in BM has been reported for other carcinoma types [16, 21, 36, 52, 91, 96]. Until now, there has

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Table 7.1. Examples of reports demonstrating a prognostic relevance of DTCs in BM of breast cancer patients without overt metastases (TNM-stage M_0)

Reference	No. of Patients	Detection Rate %	Technique	Prognostic Value
Landys et al. [69]	128	19	IHC	DFS*, OS*
Diel et al. [34]	727	43	ICC	DFS*, OS*
Mansi et al. [76]	350	25	ICC	DFS, OS
Gebauer et al. [42]	393	42	ICC	DFS*, OS
Cote et al. [28]	49	37	ICC	DFS
Braun et al. [20]	552	36	ICC	DDFS*, OS*
Harbeck et al. [47]	100	38	ICC	DFS*, OS*
Gerber et al. [43]	484	31	ICC	DFS*, OS*
Wiedswang et al. [132]	817	13	ICC	DDFS*, OS*
Braun et al. [23]	4,703	31	ICC	DDFS, OS

DFS, disease-free survival; DDFS, distant disease-free survival; ICC, immunocytochemistry; IHC, immunohistochemistry; OS, overall survival. * Prognostic value supported by multivariate analysis.

been no report of a solid tumor that does not have immunocytochemically detectable epithelial cells in BM. For example, DTCs have been found in the BM of patients with colon cancer, even though that cancer rarely metastasizes to the bone [24]. These findings indicate that BM is an important site that allows DTCs to persist and probably to disseminate to other organs.

To detect DTCs in BM, patients with breast cancer have been most thoroughly studied. These data have shown a correlation between DTCs in BM and an impaired prognosis (Table 7.1). A recent analysis of more than 4,700 breast cancer patients with stage I, II, or III disease without manifest metastases demonstrated that the presence of DTCs in BM was associated with larger tumors, a higher histological grade, lymph node metastases, and hormone-receptor-negative tumors [23]. Subgroup analysis showed that DTCs in BM are associated with worse outcomes in all risk groups. This finding has prognostic relevance even for patients with small tumors and no lymph node involvement.

A negative BM finding therefore may help identify those node-negative patients who have responded to surgery alone and do not need additional adjuvant chemotherapy [23, 75]. Several studies have reported that DTCs were found in BM even some years after surgery and adjuvant therapy. Detection of DTCs after adjuvant treatment may therefore be useful for identifying patients with an increased risk for recurrence [18, 130]. However, before DTC detection in BM can be utilized for prognosis, methods with a high degree of reproducibility must be standardized. This chapter will describe and discuss the current status of DTC detection techniques in more detail.

7.3 Potential and Limitations of DTC Enrichment and Detection Methods

7.3.1 Enrichment Methods

The unambiguous identification and characterization of DTCs requires extremely sensitive methods. The number of target cells often is so small that they cannot be detected by most analytical methods. This limitation was overcome when reagents and techniques were developed that significantly enriched the target cell population, and thus increased the sensitivity for tumor cell detection. The usual tumor cell enrichment procedures are (1) density gradients, (2) immunomagnetic procedures, or (3) size filtration [97, 103, 137, 139].

The standard Ficoll gradient separation technique or red blood lysis is often combined with positive or negative immunomagnetic cell selection. Antibody-tethered micro- or nano-sized paramagnetic beads are used to target tumor cells (as a positive selection) or unwanted hematopoietic cells (as a negative selection). The positive selection for breast cancer and other solid tumors usually applies antibodies

with magnetic microbeads against epithelial cell adhesion molecule (EpCAM), sometimes combined with anti-Her-2 antibodies. In contrast, the negative selection uses antibodies against CD45, a surface protein present on most hematopoietic cells. The positive selection procedure has limited sensitivity, because EpCAM- or HER2-negative DTCs are lost during the cell enrichment, in which case they cannot be detected thus producing false-negative findings. Expression of the selected tumor markers by normal cells (i.e., some BM cells may express EpCAM [26]) leads to the improper selection of unwanted cells. Moreover, inflammation or therapy can alter expression of epithelial or tumor-associated genes in normal hematopoietic cells [57, 58]. On the other hand, the depletion of CD45+ cells avoids stimulating targeted cells and makes it unnecessary to bind magnetic microbeads onto tumor cells. This occurrence can create a problem for subsequent analysis (e.g., cell culture, flow cytometry) and may require additional steps to dissociate the magnetic beads from the cells.

Another gradient-based enrichment of DTCs is the Oncoquick® system (Greiner Bio-One GmbH, Frickenhausen, Germany). This system employs a liquid separation medium optimized for the specific enrichment of DTC and is based only on their buoyant density under appropriate conditions. The enrichment principle does not rely on the expression of specific membrane antigens such as EpCAM or HER-2 and yields bead- and antibody-free tumor cells [84]. So far, this technique has been validated only with breast cancer cells and it is not yet known whether other epithelial tumor cells can be enriched by utilizing this method. In our hands, DTCs from prostate cancer patients are lost during Oncoquick-based enrichment (Pantel et al., unpublished).

The RosetteSep® technology (Stem Cell Technologies, Vancouver, Canada) offers another approach to obtain tumor cell-enriched preparations. The RosetteSep® antibody cocktail (tetrameric antibody complexes) crosslinks unwanted $CD45^+$ cells in human whole blood to multiple red blood cells, forming immunorosettes. This procedure increases the density of the unwanted (rosetted) $CD45^+$ cells, such that they pellet along with the free red blood cells when centrifuged over a density medium. The desired target cells are not labeled with antibody and can be easily collected in a highly enriched form while floating at the interface between the plasma and the density medium.

Finally, isolation by size of epithelial tumor cells (ISET) is based on the observation that the vast majority of peripheral blood leukocytes are among the smallest cells in the body, with a size ranging from 8 μm to 11 μm. By filtering the blood through a polycarbonate membrane with calibrated pores of 8 μm [97, 127], the leukocytes are concentrated and can be stained and/or immunolabeled. By eliminating multiple separation steps, this method minimizes cell damage and the loss of rare cells. However, experience with this method remains limited.

Development of improved enrichment procedures has been important, but it is unclear if the new enrichment techniques provide more clinically relevant information than the standard density gradient procedure (Fig. 7.1).

7.3.2 Detection Methods

The advantages and disadvantages of the most common assays that have been developed to detect DTCs in breast cancer and other types of carcinomas will be discussed in the following sections.

7.3.3 Immunocytochemical Techniques

A common approach to identify DTCs is immunocytochemical staining with monoclonal antibodies against epithelial or tumor-associated antigens (Fig. 7.1) [37, 42, 90]. The prognostic impact of immunocytochemical DTC detection at the time of primary surgery was confirmed in a large study of 4,703 breast cancer patients with a 10-year follow-up [23]. Cytokeratins (CK) are the most widely accepted protein markers to detect epithelial tumor cells in mesenchymal tissues such as BM, blood, or lymph nodes [20, 23, 94], but differences in staining techniques can create differences in antibody

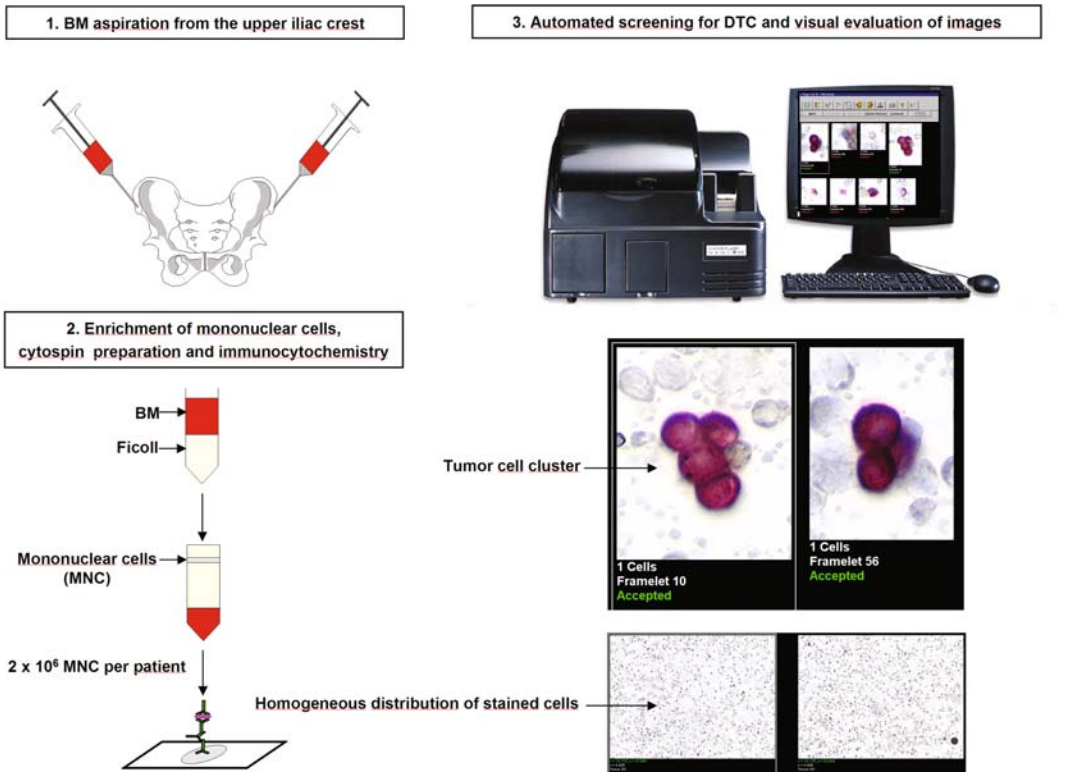

Figure 7.1. Standardized method for BM preparation and detection of disseminated tumor cells. **A**. BM aspiration from the upper iliac crest. **B**. Enrichment of mononuclear cells, cytospin preparation, and immunocytochemistry. **C**. Automated screening for DTCs and visual evaluation of images.

specificity and staining patterns [14, 17]. The assay and its evaluation therefore have been standardized on an international scale [15, 37, see also www.dismal-project.eu].

7.3.3.1 Advantages

The immunocytochemical approach provides (1) a morphological analysis of DTCs, (2) characterization of the DTCs by multiple labeling of relevant antigens, (3) direct quantification, and (4) the possibility of using the cells for further analyses (e.g., fluorescence in situ hybridization (FISH) or comparative genomic hybridization (CGH)).

7.3.3.2 Disadvantages

(1) The morphological criteria for the identification of DTCs are subjective and reliable characterization requires long-term expertise, and (2) the screening of large numbers of BM or blood cells takes a long time.

Automated devices for the microscopic screening of immunostained slides (Fig. 7.1) have made slide analysis more rapid and more reproducible [7, 13, 65, 66, 79, 135]. Of the available semi-automated systems, the FDA-approved CellSearch™ system has gained popularity because of its automated immunomagnetic enrichment and its ability to stain for cytokeratin-positive cells in blood samples [30, 108].

7.3.4 Polymerase Chain Reaction Based Assays

Molecular detection procedures have become a widely used alternative to immunocytochemical DTC assays. In principle, the nucleic acid in a sample is amplified by polymerase chain reaction (PCR), permitting a very small number of tumor cells to be detected. However, the procedure depends on changes in DNA or mRNA

expression patterns that distinguish tumor cells from the hematopoietic cells. Because the DNA of breast carcinomas is heterogeneous, there is no universally applicable DNA marker. Therefore, molecular diagnostic assays for breast carcinomas have focused on detection of RNA markers.

7.3.4.1 Advantages

(1) The molecular technique has high sensitivity, (2) it allows observer-independent detection of DTCs, and (3) quantitative real-time RT-PCR may increase discrimination between mRNA expression of normal and tumor cells, thereby increasing the specificity of the RT-PCR approach [11].

7.3.4.2 Disadvantages

(1) Inadequate expression of epithelial and/or tumor-specific mRNAs, (2) RNA instability, leading to false-negative results, (3) false-positive results attributable to specificity problems due to low-level gene expression in normal hematopoietic cells [64], which occurs particularly if a nested PCR approach is used, (4) no visualization or isolation of DTCs, (5) the heterogeneity of tumor cells may make it difficult to identify a gene that discriminates between normal and tumor cells.

Many transcripts have been evaluated as "tumor-specific" markers, e.g., cytokeratins CK18, CK19, and CK20, mucin-1 (muc-1), and carcinoembryonic antigen (CEA) [32]. When, in breast cancer, CK19 mRNA transcripts are detected in peripheral blood, survival is adversely affected [55], particularly in early-stage patients with estrogen receptor-negative tumors. However, the downregulation of CK19 mRNA marker expression [133] is an argument in favor of a multimarker RT-PCR approach [17, 70, 121].

7.3.5 Enzyme-Linked Immunospot (ELISPOT) Technology

A drawback of both immunocytochemical and RT-PCR assays is that they cannot distinguish between viable and apoptotic cells. A relatively new technique that makes this distinction is now available for DTC and CTC analyses of BM aspirates and blood samples [89]. This technique, designated as EPISPOT (i.e., epithelial immunospot), uses an adaptation of the ELISPOT technology and is based on the secretion or active release of specific marker proteins. The immunospots are due to the protein fingerprints left only by the viable secreting cells. Cell culture is needed to accumulate enough of the released marker proteins. This avoids detecting dead cells, as they do not secrete enough protein for analysis [2, 31]. Addition of cycloheximide to the cell culture on the nitrocellulose membrane causes the immunospots to disappear, a method that confirms the *de novo* synthesis of proteins. The EPISPOT assay can be combined with epithelial cell-enrichment by using a $CD45^+$ cell depletion.

7.3.5.1 Advantages

(1) The only assay that detects viable DTCs, i.e., cells that can release a marker protein, (2) secreted proteins are immunocaptured by the membrane before dilution in the supernatant. The EPISPOT assay therefore has a sensitivity that is four orders of magnitude above that when these proteins are quantified in cell-free culture supernatants [2, 4].

7.3.5.2 Disadvantages

(1) The protein used to identify a cell must be actively secreted, shed, or released from the cells, (2) at this time, there is no possibility to identify and isolate the specific protein-secreting cell(s) after cell culture.

Muc-1 and CK19 have been used as marker proteins to detect breast cancer-derived DTCs/CTCs [5]. Muc-1 is a membrane-bound mucin overexpressed and aberrantly glycosylated in breast carcinoma cells; it is cleaved, shed, and detected in the serum as a tumor marker called CA15-3 [51, 82, 95]. CK19, one of three main keratins besides CK8 and CK18, is found in simple or stratified epithelium and in carcinomas. CK19 is abundantly expressed in primary epithelial tumors, e.g., breast, colon, lung, and hepatocellular carcinoma cancer cells [27, 140], but not in normal mesenchymal hematopoietic cells. Muc-1-secreting cells (SCs) have been detected in the blood of all

advanced metastatic breast cancer patients, but not in healthy controls (3). Enumeration of both Muc-1- and CK19-SCs allows detection of viable DTCs in the BM of 90% of breast cancer patients with and 54% of breast cancer patients without overt distant metastasis [4]. These findings are comparable to those obtained with sensitive RT-PCR-based techniques [139]. Breast cancer patients in whom CK19 releasing cells have been detected are likely to have an unfavorable clinical outcome resulting from metastasis (M0-patients) or metastatic progression (M1-patients) [87].

EPISPOT technology reveals a unique fingerprint of single viable tumor cells. Because many secreted proteins modulate metastatic progression (e.g., growth factors and proteases) and because a wide range of fluorochromes is available, it should be possible to extend this technique to a multiparameter analysis. The technique therefore also should lead to an understanding of the biology of early metastatic spread. In view of the marked genetic heterogeneity of DTCs, when there is minimal residual cancer, analysis of a variety of markers and the use of several methods is recommended.

7.4 Characterization of DTCs

7.4.1 Phenotypic and Molecular Characterization of DTCs

The molecular and phenotypic characterization of DTCs remains a challenge, not only because there are so few of these cells, but also because of the difficulty in obtaining enough cells to separate and isolate without contamination by other cell types. Most DTCs markers are not tumor-specific and DTCs only can be characterized by epithelial- or organ-specific genes or proteins. To prove these cells are malignant requires further phenotypic and/or molecular characterization, a process that is hampered by the fact that DTCs exhibit features that differ from those of the primary tumor.

The purpose of characterizing DTCs is to reveal diagnostically and therapeutically relevant features that would lead to an anti-metastatic therapy that is more targeted and individualized. Fehm et al. [38] have shown, using multiple FISH analyses, that the majority of CTCs isolated from the blood of breast cancer patients are aneusomic, i.e. have deviations from the normal state of disomy, and are derived from the primary tumor. Interestingly, Schardt et al. [112] detected a DTC population with normal karyotypes that had been isolated from the BM of breast cancer patients. Nevertheless, the malignant origin of these cells was confirmed by analyzing a given cell for chromosomal aberrations, subchromosomal allelic losses, and gene amplification [112].

CGH has shown that even in early-stage breast cancer DTCs in BM are genetically heterogeneous. In advanced breast cancer, however, genetic aberrations are fewer [62, 115]. Interestingly, the early DTCs lacked some genomic aberrations found in different regions of the primary tumors [40]. This suggests that DTCs evolve independently from the primary tumor and accumulate new genomic aberrations in the course of homing to BM and other distant organs [112]. Individual CTCs isolated from the blood of prostate cancer patients may originate in distinct tumor foci within the primary tumor, usually a tumor region that represents the hormone-refractory tumor fraction [114]. In primary tumors, loss of heterozygosity in distinct markers, as in those representing the tumor suppressor genes PTEN and BRCA1, seems to be associated with the occurrence of CTCs in prostate cancer patients [114]. DTCs in BM are found in up to 90% of patients with active prostate cancer. Most of these DTCs express PSA, with the highest numbers in refractory cancer patients that had been treated with androgen ablation therapy [99].

DTCs are heterogeneous with respect to the expression of growth factor receptors, adhesion molecules, proteases and their inducer and receptors, major histocompatibility complex antigens, or signaling kinases [50, 60, 63, 80, 92, 107, 113, 122]. The epidermal growth factor receptor HER2 is of particular interest. When this marker is found to be overexpressed in the primary tumors of breast cancer patients, treatment with trastuzumab (Herceptin®) is usually prescribed. HER2 overexpression of DTCs from

BM predicts a poor clinical outcome for stage I–III breast cancer patients [22]. Schardt et al. [112] identified a subset of cytokeratin-positive, CGH-normal DTCs from BM of early M0-stage breast cancer patients who displayed HER2 gene amplification. In most other cases HER2 gene amplification in DTCs occurs later in cancer progression [112]. Even though it was shown that in one set of breast cancer patients the HER2 status remained relatively stable between primary tumors and BM micrometastases [126], there also is increasing evidence [112, 119] that there is a discrepancy between the HER2 status in primary tumors and that in DTCs in BM. Even though HER2 expression does not fully parallel that in DTCs from specific patients, it is conceivable that, if DTCs are HER2-positive in patients whose primary tumors are not HER2-positive, Herceptin therapy may be indicated [119]. Whether HER2-positive DTCs reflect the primary tumor and/or metastases and are an indication for Herceptin treatment is not known.

Kufer et al. [67] analyzed BM aspirates from 106 patients with breast, lung, colorectal, prostate cancer, and different sarcomas. With the aid of the multimarker RT-PCR, these investigators found different MAGE-A (melanoma-associated antigen) genes expressed in a variety of malignancies, whereas 30 BM samples from healthy donors were completely MAGE-A-negative. MAGE-A expression, determined by the multimarker RT-PCR assay, overlapped significantly with positive results of the cytokeratin-based test [67]. Prostate cancer patients with a high risk of metastatic relapse were more frequently MAGE-A-positive than patients with a lower risk [67]. The recently developed multimarker real-time PCR assay for the detection of MAGE-A gene expression in blood and BM is sufficiently sensitive to detect and quantify the minimal systemic tumor load in prostate cancer patients with localized disease [78]. Sienel et al. [118] have shown that the presence of MAGE-A transcripts can serve as an independent prognostic factor for patients with operable non-small cell lung cancer.

Approximately 50% of DTCs from prostate cancer patients express telomerase activity, potentially predictive for early recurrence [98]. Telomerase activity did not correlate with Gleason-score, pre-operative PSA level, tumor stage, or surgical margin status [98]. On the other hand, telomerase-negative DTCs may be dormant, inasmuch as telomerase is a marker for cell proliferation in cancer cells [98].

Microarray-based gene expression analyses of primary breast tumors from patients with DTCs in BM [85, 136] support the hypothesis that tumor cells acquire the genetic changes relevant to their metastatic capacity early in tumorigenesis [12]. Accordingly, the metastatic potential of DTCs already may be encoded in most primary tumor cells [12, 106]. Information about the overall gene expression program of DTCs is limited. Watson et al. [128] reported that DTCs in BM after chemotherapy are characterized by unique transcriptional signatures. TWIST1, a transcription factor, plays an important, role in metastasis by promoting epithelial–mesenchymal transition [25, 61, 74, 111]. TWIST1 has been identified as part of the gene expression signature in EpCAM-enriched cells from BM of breast cancer patients after chemotherapy. The upregulated expression of TWIST1 correlated with the occurrence of distant metastasis and local progression, even in pretreatment BM samples [128], and was not observed in EpCAM-enriched cells of BM obtained from healthy volunteers. Studies are needed to elucidate if individual DTC-specific genes are markers for the prediction of early recurrence in breast cancer [128].

7.4.2 Features Enabling Homing, Dormancy, and Re-growth of DTCs

Absence of the proliferation marker Ki-67 in DTCs indicates that most DTCs in BM are non-proliferating and dormant [84, 93]. This may explain why adjuvant chemotherapy is ineffective [19]. Because only half of the breast cancer patients with DTCs relapse, while the other half remain tumor-free for 10 years [23], it seems likely that a significant fraction of DTCs remains dormant and does not metastasize. When DTCs are detected in BM even years subsequent to primary breast cancer treatment, the risk of late metastatic relapse has increased [56, 130]. To overcome dormancy and then to

metastasize must involve both genetic and epigenetic changes in the DTCs, i.e., additional mutations, and/or methylation/demethylation in the DTCs. Changes in the microenvironment or premetastatic niche, i.e., stress, immune surveillance, dietary changes, increased angiogenetic potential, etc. [77, 86, 125] [see Chapter 1 of this volume] also may contribute to disease recurrence. Crucial conditions and timing for outgrowth of dormant tumor cells are not known [3, 88]. Furthermore, primary tumor cells need to be analyzed for the factor(s) that enable them to lodge in BM. In esophageal cancer, the expression in primary tumors of the CXC-chemokine receptor, CXCR4, parallels the presence of DTCs in lymph nodes and BM [59]. Tumor cells with a metastatic potential may use this chemokine-mediated mechanism to home to BM, a tissue rich in the stromal cell-derived chemokine, SDF-1, which acts as ligand for CXCR4 [83]. Other proteins needed by DTCs for transition from dormancy are HER2 and the urokinase-type plasminogen activator receptor (uPAR). The overexpression of HER2 in CTCs from blood and DTCs from BM correlates with metastatic relapse in breast cancer [138] and that of uPAR with relapse in gastric cancer [49]. Strategies to induce or maintain tumor cell dormancy may involve blocking these proteins [1].

Host hereditary factors, in particular host genetic polymorphisms, can modulate the metastatic efficiency of tumor cells [29, 53, 54]. Further research is needed to identify genetic polymorphisms that are associated with an increased capacity of DTCs to survive and to escape from dormancy.

7.4.3 Stem Cell-Like Phenotypes of DTCs

The concept that cancer originates from stem or progenitor cells that can self-renew and differentiate has received growing attention as a result of the discovery of new stem cell markers and signatures [104, 105, 116, 120, 129]. These markers include those that occur in many tumors, as well as others found only in a limited number of tumors [116]. CD133, Bmi-I, or Oct-4 are commonly expressed cancer stem cell markers [116]. The presence of CD44 and the absence of low-level expression of CD24 characterize breast cancer "founder" cells. Founder cells can produce more tumors in immunosuppressed mice than do other breast cancer cells [39, 100, 117]. Interestingly, microarray gene expression analysis of the ratio of $CD44^+/CD24^{-/low}$ cells (in the absence of $CD44^-$ cells) in breast cancer tissue produced a 186-gene signature of invasion that was significantly associated with overall and metastasis-free survival of breast cancer patients [72].

Ginestier et al. [44] reported that the expression of aldehyde dehydrogenase (ALDH1), a new stem cell marker, was associated with poor clinical outcome in breast cancer. Furthermore, highly ALDH-1-active breast cancer cells are strongly tumorigenic when transplanted as xenografts in NOD/SCID mice. These cells self-renew and generate tumors that recapitulate the heterogeneity of the parental tumors [44].

The "founder" cells of overt metastases may represent "metastatic stem cells" among the DTCs, because DTCs in BM and metastatic relapse have been shown to be positively and significantly correlated [23]. Moreover, most DTCs are non-proliferative, have long persistence, and are resistant to conventional systemic chemotherapy [8, 9, 20, 84, 93]. This also is true for tumor stem cells [33, 46]. Accordingly, when genetic or environmental changes cause DTCs to no longer be dormant, metastatic relapse may ensue.

Recently it was shown that most DTCs in BM were $CD44^+/CD24^{-/low}$ and expressed stem cell features [6]. A proportion of viable DTCs enriched from BM of metastatic and primary breast cancer patients present with a breast cancer stem cell-like phenotype [5], characterized by CK19-positive status and the absence of Muc-1 secretion [45]. Whether DTCs can self-renew, as do stem cells, remains to be elucidated.

The pathways that confer tumor stem cells with chemoresistance, i.e., influence proliferation and differentiation, suppress apoptosis, or increase expression of drug transporters or DNA repair enzymes, remain under investigation [81]. Aside from the Notch signaling pathway in breast cancer stem cells [35, 81], developmental pathways such as Wnt and hedgehog also contribute to regulating cancer stem cell features

[71, 73, 81]. If these pathways could be targeted, it might be possible to eradicate tumor stem cells, thereby minimizing the chance of developing metastases.

7.4.4 Identification of Therapeutic Targets for DTCs

If DTCs in fact differ genetically and phenotypically from the corresponding primary tumors, DTC characterization could identify particular cancer patients for targeted therapies. In breast cancer patients, the most prominent therapeutic target is HER2, which is overexpressed in 20–30% of these patients. Currently, breast cancer patients are treated with human anti-HER2 monoclonal antibody trastuzumab [101, 110], based only on analysis of the phenotype of the primary tumor. However, HER2-positive DTCs or CTCs now are known to be present also in patients with HER2-negative primary tumors [119, 138], an observation that is consistent with the high frequency and prognostic relevance of HER2 expression in BM [22]. More patients therefore may benefit from HER2-targeting therapies [119]. Ongoing clinical studies will show whether the HER2 status of DTCs correctly predicts the response to trastuzumab or other HER2-directed therapies.

Most DTCs that remain in 50% of BM samples from ovarian cancer patients after platinum-based chemotherapy co-express cytokeratin and EpCAM and are non-apoptotic. Ovarian cancer patients therefore may benefit from therapy based on an antibody that targets EpCAM [134]. Other molecules of interest in anti-cancer therapies are antigen-like MAGE (see above), molecules that are frequently expressed not only in different primary tumors, but also in DTCs from a variety of cancers [67]. Because MAGE antigens can induce autologous cytolytic T-lymphocytes in vitro [41, 123, 124], the identification of individual expression patterns of MAGE genes in cancer patients may help develop anti-tumor vaccines [67].

Analysis of BM for DTCs during and after systemic adjuvant therapy following surgery may provide unique information on managing cancer patients [8, 9, 20, 56, 130] at risk of recurrence after first-line therapy has been completed. These patients may benefit from an additional or "second-line" therapy, as with bisphosphonates or treatment targeted at growth factor receptors or angiogenesis. Monitoring studies require repeated BM sampling, but BM aspiration is invasive, time-consuming and, in many cases, painful or uncomfortable. CTC detection in the peripheral blood therefore has been attempted [109]. Studies comparing simultaneous BM and peripheral blood samples, though limited in number, have shown that peripheral blood samples have lower prediction power [10, 84, 102, 131]. Conceivably BM, by providing conditions for DTC homing and survival, attracts more tumor cells. Nonetheless, blood samples may provide supplementary data, particularly if the analysis is repeated [48, 68].

In summary, progress has been made in detecting and characterizing DTCs in BM of patients with solid tumors. This information may improve treatment strategies, particularly if the new knowledge can be translated into new therapeutic options for the individual patient.

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8.

Molecular Imaging of Cancer Cells Growing in Bone

Inna Serganova and Ronald G. Blasberg

8.1 Introduction and Background

Recently, a definition of molecular imaging was recommended by the Molecular Imaging Center of Excellence (MICoE) and the Society of Nuclear Medicine (SNM). Molecular imaging was defined as "the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in humans and other living systems". Although the term "molecular imaging" was coined in the mid-1990s, it has its roots in molecular and cell biology, as well as in imaging technology, chemistry, and radiochemistry. Molecular imaging is the direct result of significant developments in several noninvasive, in vivo imaging technologies including magnetic resonance imaging (MRI) [59, 114], nuclear imaging (gamma camera and a positron emission tomography (PET)) [6], and optical imaging of small animals [36, 57, 112] (Fig. 8.1).

8.2 Molecular Imaging Strategies

The most widely used molecular imaging modalities include (1) optical (fluorescence, bioluminescence, and spectroscopy), (2) radionuclide (PET, SPECT (single-photon emission computed tomography), gamma camera, and autoradiography), (3) magnetic resonance (spectroscopy, contrast, diffusion-weighted imaging), (4) ultrasound, and (5) computed tomography (CT). Before discussing specific molecular imaging issues and application in the imaging of cancer cells growing in bone, it will be helpful to outline briefly three current imaging strategies to monitor and measure molecular events noninvasively. These strategies have been broadly defined as "biomarker", "direct", and "indirect" imaging.

8.2.1 Biomarker Imaging

Biomarker imaging can be used to assess downstream effects of one or more endogenous molecular-genetic processes. This approach is particularly attractive for potential translation into clinical studies in the near term, because existing clinical imaging paradigms may be useful to monitor changes in specific molecular-genetic pathways in cancer. For example, imaging a tumor on the basis of its glucose utilization can be done by using a radiolabeled analog of glucose (2′-fluoro-2′-deoxyglucose – [^{18}F]FDG) and PET. This is possible because malignant

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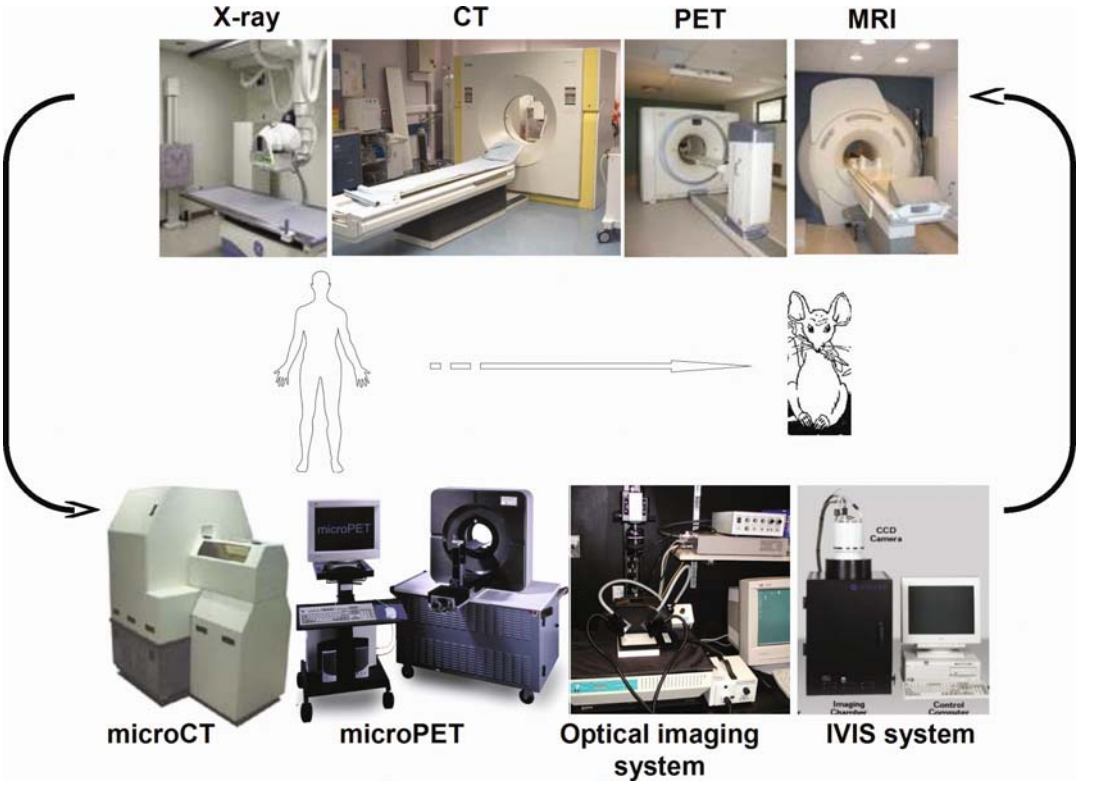

Figure 8.1. From men to mice. The imaging technologies developed for human application were converted for use in mouse models. In addition to X-ray, CT, PET, and MRI, new instrumentation has appeared for in vivo imaging of fluorescence and bioluminescence reporters in mice (e.g., IVIS imaging system and fluorescence imaging systems, based on a CCD camera for image acquisition).

tumors frequently have high glycolytic rates [109]. [^{18}F]FDG PET also is used to assess biological effects that occur during neoplastic progression or to monitor the efficacy of anti-cancer therapies [63, 96, 105] (Fig. 8.2).

Radionuclide bone scanning with technetium 99m (^{99m}Tc) methyl diphosphonate has been the standard for evaluating individuals who may have bone metastases. [^{18}F]FDG PET, however, may provide comparable accuracy [22, 23]. Integrated PET/CT can help differentiate between FDG-avid lesions that are located within bone versus those in adjacent soft tissue. CT–FDG PET co-registered images provide added value in the assessment of bone cancer and metastases. Several studies have been performed to evaluate how [^{18}F]FDG PET contributes to the identification of malignant bone lesions [34, 60, 87]. [^{18}F]FDG biomarker imaging has proven very useful for the clinical management of individual patients, even though it is relatively "non-specific," and likely to implicate more than one protein or signaling pathway.

8.2.2 Direct Molecular Imaging

Direct imaging strategies usually are described in terms of a specific target or target-specific probe and utilize nuclear, optical, and MR imaging technology. The resultant image of probe localization and concentration (signal intensity) is a direct function of its interaction with the target. Imaging cell surface-specific antigens with radiolabeled antibodies and genetically engineered antibody fragments, such as minibodies, is one example of direct molecular imaging that has evolved over the past 30 years. More recent research has focused on the chemistry and synthesis of small radiolabeled or fluorescent molecules that target specific receptors, for example, the estrogen or androgen receptors

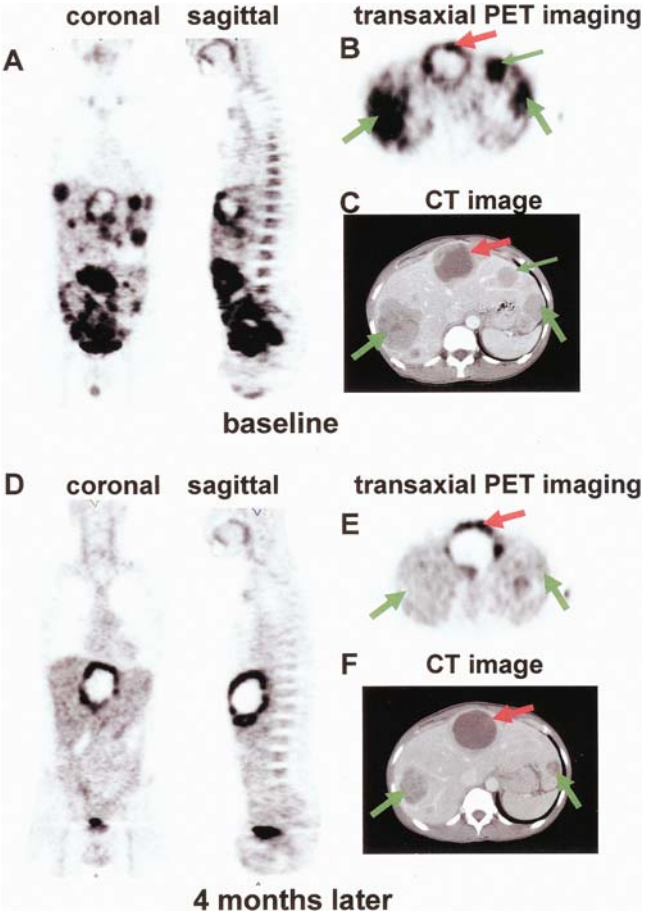

Figure 8.2. [^{18}F]FDG PET imaging of gastrointestinal stromal tumors (GISTs). [^{18}F]FDG PET and CT scans of patient with metastatic GIST in the abdomen and liver, before (**A, B, C**) and after 4 months of therapy with STI-571 (Gleevec) (**D, E, F**). After treatment, CT images show that several of the tumors are still present and have decreased very little in size. [^{18}F]FDG PET imaging shows a marked decrease in glucose utilization in all but one tumor after treatment with STI-571 (Gleevec). Figure provided by Henry Yeung and Tim Akhurst with appreciation and permission, Memorial Sloan-Kettering Cancer Center, New York, NY.

[31, 71] and fluorescent probes that are activated by endogenous proteases [64]. For example, the $\alpha_v\beta_3$ integrin is highly expressed in tumor vasculature and plays an important role in metastasis and tumor-induced angiogenesis. Initial studies of targeting and imaging of the $\alpha_v\beta_3$ integrin with radiolabeled glycosylated RGD-containing peptides are very encouraging (Fig. 8.3) [52]. Another example is direct imaging of the cell surface receptor tyrosine kinase HER2, which is overexpressed in many breast tumors. The level of expression of HER2 can be imaged with radiolabeled [7, 43, 83] or gadolinium-chelated [3] antibodies specific for HER2. The major limitation of direct radiotracer imaging strategies is the necessity to develop a specific probe for each molecular target, and then to validate the sensitivity, specificity, and safety of each probe. This can be very time-consuming and costly. For example, the development, validation, and regulatory approval for [^{18}F]FDG PET imaging of glucose utilization in tumors has taken over 20 years.

8.2.3 Indirect (Reporter Gene) Molecular Imaging

Indirect imaging strategies are more complicated. One example of indirect imaging now

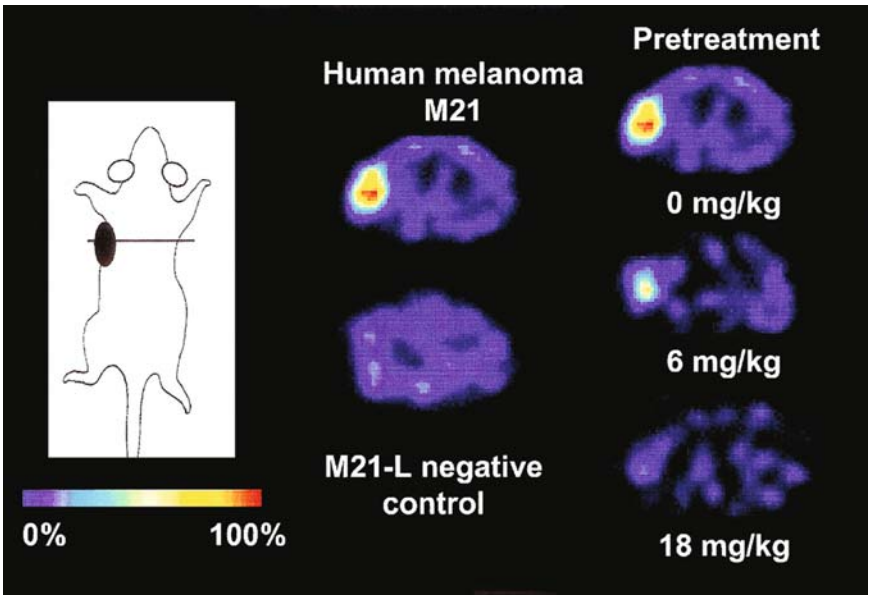

Figure 8.3. Noninvasive imaging of $\alpha_v\beta_3$ integrin expression by PET. Transaxial PET images of nude mice bearing human melanoma xenografts. Images were acquired 90 min after injection of approximately 5.5 MBq of [^{18}F]Galacto-RGD. The *top left* image shows selective accumulation of the tracer in the $\alpha_v\beta_3$-positve (M21) tumor on the left flank. No focal tracer accumulation is visible in the $\alpha_v\beta_3$-negative (*M21-L*) control tumor (*bottom left image*). The three images on the *right* were obtained from serial [^{18}F]Galacto-RGD PET studies in one mouse. These images illustrate the dose-dependent blockade of tracer uptake by the $\alpha_v\beta_3$ integrin-selective cyclic pentapeptide cyclo (-Arg-Gly-Asp-D-Phe-Val-). Figure adapted with permission from Haubner et al. [52].

being used widely is reporter gene imaging. It requires "pre-targeting" (delivery) of the reporter gene to the target tissue (by transfection/transduction), and usually includes transcriptional control components that can function as "molecular-genetic sensors" that initiate reporter gene expression. This strategy has been widely applied in optical [21, 57, 90] and radionuclide-based imaging [45, 102, 103, 104] and to a lesser degree for MR [72, 114] imaging. Early reporter gene imaging approaches required post-mortem tissue sampling and processing [41, 82]. A general paradigm for "noninvasive reporter gene imaging" using radiolabeled probes was first described in 1995 [104] and is diagrammed in Fig. 8.4**.** This paradigm requires the appropriate combination of a reporter transgene and a reporter probe. The reporter transgene product, usually an enzyme, transporter, or receptor selectively interacts with a specific radiolabeled probe and accumulates only in transduced cells (Fig. 8.4A–C). Alternatively, the enzyme (e.g., luciferase) catalyzes a reaction that yields light (photons) in the presence of substrate (e.g., luciferin). The reporter gene product can itself be fluorescent, allowing it to be imaged in vivo or ex vivo (Fig. 8.4D).

This Chapter will describe the development of reporter genes for noninvasive quantitative imaging in small animals and the potential for translation to patient care. The description of PET/SPECT-based reporter imaging genes and their complementary probes will be followed by characterization of other reporter genes, mostly for optical imaging, that have been developed for work with xenografts and transgenic animal models of cancer.

8.2.3.1 Radiotracer Reporter Gene Imaging

8.2.3.1.1 Herpes Simplex Virus 1 Thymidine Kinase Gene

The *herpes simplex virus type 1 thymidine kinase* (HSV1-*tk*) gene has gained considerable attention as a "suicide gene" in clinical medicine and as a radiotracer-based reporter gene. The HSV1-TK enzyme, like mammalian thymidine

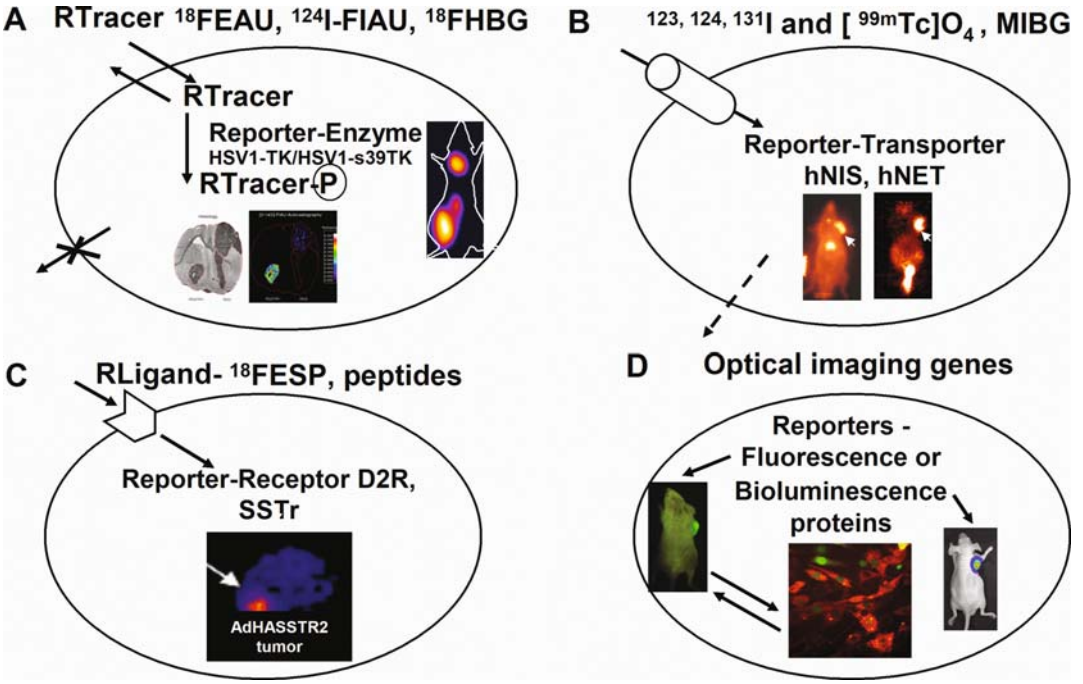

Figure 8.4. The reporter imaging genes and their tracers. Diagrammatic presentation of a general paradigm for noninvasive reporter gene imaging, using radiolabeled probes and fluorescence/bioluminescence imaging. This paradigm requires the appropriate combination of a reporter transgene and a reporter probe. The reporter transgene product (a protein or enzyme) selectively interacts with a specific radiolabeled probe and results in its accumulation in transduced cells only. Alternatively, the enzyme (e.g., luciferase) will catalyze a reaction to yield light (photons) in the presence of substrate. The reporter gene product could also be a fluorescent protein that can be imaged in vivo as well as ex vivo.

kinases (TKs), phosphorylates thymidine to thymidine monophosphate (TdR). Unlike mammalian TKs, viral HSV1-TK can also phosphorylate modified thymidine analogs, including 2′-deoxy-2′-fluoro-5-iodo-1-[β]-D-arabinofuranosyluracil (FIAU), 2′-fluoro-5-ethyl-1-[β]-D-arabinofuranosyluracil (FEAU) as well as acycloguanosine analogs (e.g., acyclovir (ACV); ganciclovir (GCV); penciclovir (PCV)) that are not (or minimally) phosphorylated by eukaryotic TKs [44]. In the mid-1990s, a number of potential marker/reporter probes for imaging HSV1-*tk* gene expression were studied at Memorial Sloan-Kettering Cancer Center (New York, NY, USA) (Fig. 8.4A). FIAU had been previously radiolabeled for imaging viral infections [94]. In vitro assays comparing different thymidine and acycloguanosine analogs for HSV1-TK sensitivity and selectivity showed that FIAU had good imaging potential. A variety of radionuclides (^{11}C, ^{124}I, ^{18}F, ^{131}I, ^{123}I) can be used for FIAU labeling. Using QAR imaging techniques, experiments involving HSV1-*tk* transduced tissue and FIAU were first performed in rats bearing intracerebral (i.c.) RG2 tumors [103, 104] (Fig. 8.4A). This was subsequently followed by gamma camera, SPECT, and PET imaging studies [102, 103]. A phase I/II clinical trial of HSV1-*tk* "suicide" gene therapy for recurrent glioblastoma indicated that PET-imaging of HSV1-TK expression in patients is feasible and that vector-mediated gene expression can predict a therapeutic effect [62].

PET imaging of HSV1-TK expression also can be performed with acycloguanosine derivatives that show very low affinity to mammalian TK-1. Radiolabeled with fluorine-18 ($t_{1/2}$ =110 min), these compounds can be used for repetitive imaging studies (every 6 to 8–12 h). After several years of comparative studies [44, 45, 61], a new radiolabeled acycloguanine, 9-(4-[^{18}F]fluoro-3-hydroxymethylbutyl)guanine or [^{18}F]FHBG [1, 117] was developed. In parallel, a mutant variant of HSV1-TK (HSV1-*sr39TK*) gene

exhibited higher uptake of [^{18}F]FHBG and greater efficacy than wild-type HSV1-TK [46]. Cells transfected with HSV1-*sr39tk* gene accumulated [^{18}F]FHBG approximately twice as well as cells expressing wild-type HSV1-TK; the resulting [^{18}F]FHBG/HSV1-sr39TK images were therefore improved. As a radiotracer, [^{18}F]FHBG has been studied in normal human volunteers and has been found to be safe and of potential value in human applications [118]. Recently, the same [^{18}F]FHBG/HSV1-sr39TK PET imaging system has been used to monitor TK gene expression after intratumoral injection of a first-generation recombinant adenovirus in patients with hepatocellular carcinoma. Transgene expression in the tumor was dependent on the injected dose of the adenovirus and was detectable by PET during the first hours after administration of the radiotracer in all patients who had received $\geq 10^{12}$ viral particles. Expression of the transgene was not detected in any distant organs, or in the surrounding liver tissue. PET imaging can therefore help in the design of gene-therapy strategies and in the clinical assessment of new-generation vectors [84, 85].

8.2.3.1.2 Sodium Iodide Symporter Gene

Radioactive iodide (^{131}I) therapy is used to treat thyroid cancer in humans, because the sodium iodide symporter (NIS) present in their plasma membranes enables thyrocytes to accumulate iodine [26]. NIS is an intrinsic membrane glycoprotein with 13 putative transmembrane domains that transport two sodium ions with one iodide ion across the cell membrane [39]. NIS can transport many other anions coupled with sodium, such as ClO_3^-, SCN^-, NO_3^-, Br^-, TcO_4^-, ReO_4^- [106]. Since 1996, when the NIS gene was cloned [26], NIS has been proposed as an imaging reporter gene [8, 16, 49] (Fig. 8.4B). Using human NIS as a reporter gene has several distinct advantages. First, hNIS is not a foreign gene; thus, it is nonimmunogenic. Second, endogenous NIS protein expression is limited to a few tissues. Therefore, exogenous NIS function can be imaged in many tissues other than stomach or thyroid. Third, hNIS mediates the uptake of simple radiopharmaceuticals; therefore, complicated syntheses and labeling of substrate molecules is not required for imaging. Fourth, because most radiotracers are specific to hNIS-expressing cells, the background signal is significantly reduced. Finally, hNIS-mediated radiotracer uptake is rapid and background radioactivity is low, making this procedure ideal for noninvasive imaging.

8.2.3.1.3 Norepinephrine Transporter Gene as a Reporter Gene

In recent years, several groups including ours have evaluated the norepinephrine transporter (NET) as a potential reporter gene for in vivo imaging [2, 14, 79]. NET is a transmembrane protein that consists of approximately 600 amino acids and mediates the transport of norepinephrine, dopamine, and epinephrine across the cell membrane. The expression of hNET is restricted to the central and peripheral sympathetic nervous system (brain, heart) [37, 70, 125]. The main advantage of using *hNET* as a reporter gene is that NET expression can be imaged efficiently using meta-iodobenzylguanidine (MIBG), a clinically approved, metabolically stable artificial analog of norepinephrine. MIBG can be readily labeled with any of the four iodine radioisotopes (^{123}I, ^{124}I, ^{125}I, ^{123}I) (Fig. 8.4B). The MIBG tracer was developed in 1980 for imaging the peripheral sympathetic nervous system [115] and subsequently was used for the diagnosis of tumors that originate in the sympathetic nervous system (neuroblastoma and pheochromocytoma). Because of its favorable biodistribution, MIBG can be readily used for whole-body imaging; unbound tracer is rapidly excreted, largely via the kidney [108]. Furthermore, MIBG uptake is highly specific for NET expression. The NET gene cloned from human tissue is nonimmunogenic and therefore is a good candidate for clinical applications and noninvasive monitoring of gene- and cell-based therapy.

The "transport class" of reporter systems has an additional advantage for molecular/reporter imaging. In contrast to the enzyme-based reporter systems, the transporters (reporter proteins) are expressed on the cell surface. This precludes concerns about intracellular accessibility of the reporter probe. This does not mean, however, that localization of the transporter (or a receptor) to the cell surface is a simple

process. Functionality of the reporter gene product involves post-translational intracellular trafficking and proper cell membrane expression, a situation likely to be altered under different conditions and disease states. In any case, it is necessary to show that the level of probe accumulation is proportional to the level of gene and is not a result of post-translational folding, intracellular trafficking, or cell membrane insertion.

8.2.3.1.4 Genes Encoding Receptors

One of the most attractive reporters from this class of reporters are the somatostatin receptors (SSTRs). Somatostatin, originally detected in the hypothalamus of rats, is a peptide that inhibits the release of growth hormone. It was subsequently shown to be a 14-amino-acid cyclic peptide [10, 68]. Somatostatin modulates neurotransmission in the central nervous system (as a neurotransmitter) and regulates the release of growth hormone and thyrotropin (as a neurohormone). The various actions of somatostatin are mediated through specific membrane SSTRs that are present in various regions of the brain, the endocrine and exocrine pancreas, and the mucosa of the gastrointestinal tract, as well as in cells of the immune system [91]. There are six SSTRs (types 1, 2A and 2B, 3, 4, and 5) and they all belong to a seven-transmembrane domain family of receptors associated with G-proteins. The type 2 SSTR (SSTR2) is expressed primarily in the pituitary gland. It has high affinity for octreotide, the first synthetic somatostatin analog introduced for clinical use. SSTR2 inhibits the release of growth hormone, glucagon, and insulin more effectively than somatostatin-14 [4]. Octreotide is more stable and resistant to in vivo degradation than the endogenous 14-amino-acid somatostatin peptide and can be labeled with ^{111}In, and isotopes that have been used for gamma-camera and SPECT imaging of endogenous SSTR2 expression in tumors [38, 76]. Intratumoral injection with an adenovirus or intraperitoneal injection with a vaccinia virus carrying the *SSTr2* gene have been successfully used with radiolabeled somatostatin analogs ^{111}In-diethylenetriaminepentaacetic acid-octreotide (Octreoscan; Mallinckrodt), ^{99m}Tc-P829 (NeoTect; Diatide, Inc.), and ^{99m}Tc-P2045 (Diatide, Inc.) to localize tumors in mice [126, 127] (Fig. 8.4C).

The dopamine receptor it is another receptor, which can be visualized because of the interaction with 3-(2′-[^{18}F]fluoroethyl)spiperone (FESP), a radiolabeled probe that was developed to image dopaminergic neurons [95]. Five subtypes of dopamine receptors, D1–D5 have been identified. They are members of a seven-transmembrane-spanning heterotrimeric GTP-binding protein (G protein)-coupled receptor (GPCR) family. The D2 receptor is a 415-amino-acid protein with maximum expression in striatum and pituitary [13]. Attempts at inducing a high level of membrane protein expression as reporter gene products face the problem of retaining function while assuring proper protein folding, intracellular trafficking, and insertion into the plasma membrane.

8.2.3.2 Optical Imaging Genes

Optical-based (bioluminescence and fluorescence) reporter systems have received increased attention because of their sensitivity, ability to bring about sequential imaging, operational simplicity, and substantial cost benefits (Fig. 8.4D).

8.2.3.2.1 Bioluminescence Imaging

Bioluminescence imaging (BLI) is based on the detection of visible light produced during enzymatic (luciferase) oxidation of a specific substrate (luciferin). Bioluminescence-based reporter systems are used extensively for in vitro assays and whole-body imaging of small animals [116, 20, 21, 51, 66, 78]. Luciferin and luciferase are generic terms, since the different luciferases do not exhibit sequence homology. The most commonly used bioluminescence reporters for research purposes have been Firefly and Renilla luciferases. Useful luciferases have also been cloned from jellyfish (*Aequorea*), sea copepod (*Gaussia*; GLuc), corals (*Tenilla*), click beetle (*Pyrophorus plagiophthalamus*), and several bacterial species (*Vibrio fischeri*, *V. harveyi*). Most commonly used reporter genes have been modified for optimum usage in mammalian cells and have been termed "humanized" variants of luciferases.

The cloning of the *Firefly luciferase* gene in 1985 has made this reporter system a useful

tool for in vitro and in vivo studies of gene regulation. Firefly luciferase (FLuc) produces photons only when oxygen, ATP, magnesium, and the substrate D-luciferin are present. Another common bioluminescence system is Renilla luciferase and its substrate coelenterazine that acts like luciferin. When oxidized by the appropriate luciferase, coelenterazine produces carbon dioxide, coelenteramide, and light. Renilla and the later identified and cloned Gaussia luciferases are the best characterized proteins that can be used with coelenterazine for BLI. The humanized form of Gaussia luciferase is a small monomeric protein composed of 185 amino acids (19.9 kDa) with a short coding sequence (555 bp). It is nontoxic and naturally secreted in the culture medium. hGLuc is heat stable and resists changes in pH. hGLuc generates a 1000-fold stronger signal from cells in culture than does hRLuc or hFLuc. Even though a significant proportion of hGLuc is secreted, its bioluminescence, whether from whole cells or a cell lysate, is 100-fold more intense than due to hRLuc or hFLuc [101]. Nevertheless, luciferases that utilize coelenterazine as a substrate have two drawbacks. First, coelenterazine presence is associated with substantial autoluminescence in the presence of certain proteins, particularly albumin [124]. Second, the MDR1 transporter can rapidly export coelenterazine from cell lines that express this protein; this results in a reduced photon emission flux [88].

The main benefit of optical BLI is its extremely low background. This means it can be used to detect very low signal levels from as few as ≈300 cells. However, light absorption and scatter vary in different tissues with emission wavelength and depth from the surface. Therefore, light transmission through the body tends to be limited.

8.2.3.2.2 Fluorescence Imaging

In the last 10 years, green fluorescent protein (GFP) has evolved from an almost unknown protein to a common reporter protein broadly used in molecular and cell biology. The fact that the GFP chromophore (formed by autocatalytic cyclization) does not require a cofactor or substrate makes this reporter particularly useful. Fluorescent protein-based reporter gene systems originated with different spectral-shifted variants of *Aequorea victoria* GFPs [40, 50, 69, 74]. Two reviews have described fluorescent protein variants, their properties, advantages, and limitations [17, 122]. Fluorescence imaging is useful for these applications: (1) monitoring gene expression, (2) tracking of proteins of interest: localization, movement, interaction, and functional activity in the cell, (3) identifying and selecting transduced cells by FACS analysis and sorting, (4) tracking the movement of labeled cells, proteins, and different organelles [18], and (5) cost-effective in vitro assays for validation of the function and sensitivity of specific inducible reporter systems. In addition, fluorescence whole-body imaging may become a key technology to visualize reporter gene expression [56, 81], interactions between proteins [29], or target gene promoter regulation [123]. An important advantage of fluorescence imaging is that tissue sections can be examined at the microscopic level [33, 56, 57]. However, fluorescence imaging also has limitations. Both transmitted and emitted light are attenuated and scattered by tissue. This significantly limits the resolution and depth of body imaging. Endogenous autofluorescence diminishes imaging quality and increases background fluorescence that in turn limits the sensitivity and specificity of imaging. Background fluorescence can be addressed by the use of selective filters or the application of spectral analysis. However, the light penetration problem is more difficult to solve. A possible solution is new classes of red fluorescent proteins and near-infrared dyes which have better deep-tissue imaging characteristics [15, 48, 119, 120]. Also, fluorescence imaging requires an external light source to activate the chromophore. This is not true for BLI.

8.3 Imaging of Cancer Cells Growing in Bone

8.3.1 Clinical Application

Bone cancers can be divided into two groups. The first includes primary bone cancers. These are extremely rare neoplasms and account for

less than 0.2% of all cancers. Primary bone cancers are characterized by clinical heterogeneity. The three most common forms of primary bone cancers are osteosarcoma (35%), chondrosarcoma (30%), and Ewing's sarcoma (16%). The second, much larger group, comprises primary tumors in other organs that have metastasized to bone. Of these, prostate, breast, and lung cancers are the most common in adults and the most common that metastasize to bone [19]. Carcinomas of kidney and thyroid and melanomas are other frequent tumors that metastasize to bone [107].

Primary bone and some metastatic tumors are diagnosed by conventional radiography, the gold standard for bone cancer evaluation since the discovery of X-rays in 1895 by W.C. Roentgen. X-rays provide high resolution, are relatively low cost, and remain the most widely used imaging modality in clinical medicine. Other methods useful in detecting bone tumors include scintigraphy (isotope bone scan), CT, and MRI: these complement conventional radiography.

8.3.1.1 Scintigraphy

The procedure of administering a radionuclide and recording the distribution of radioactivity by an external scanning scintillation camera is named scintigraphy. Bone-seeking agents like a technetium (^{99m}Tc)-labeled diphosphonate accumulate mostly in regions with increased blood flow and new bone formation [35] (Fig. 8.5). Scintigraphy is more suitable for the detection of metastatic disease, especially in the early phases, than for the detection of primary

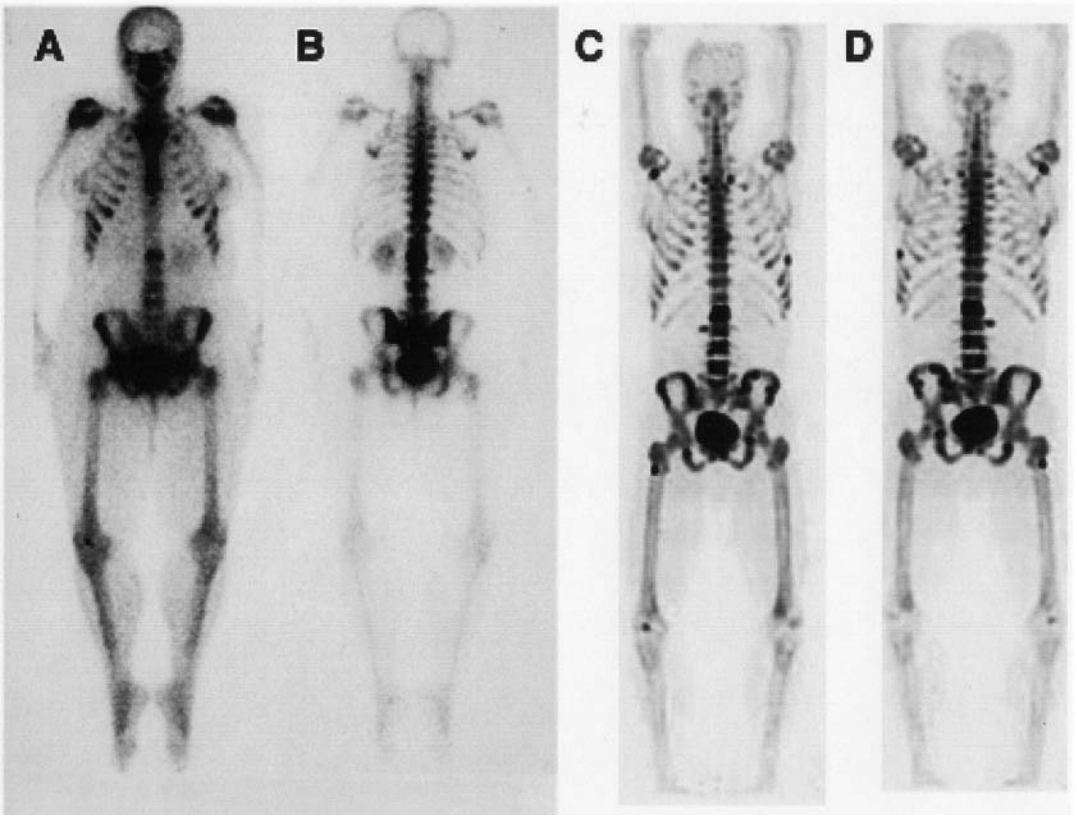

Figure 8.5. Bone scans. A [^{99m}Tc]-methylene diphosphonate (MDP) planar bone scan and a [^{18}F] fluoride bone scan of same patient obtained within a few days of each other. (**A** and **B**) Anterior and posterior views showing increased [^{99m}Tc]MDP uptake in some vertebra and ribs. (**C** and **D**) Anterior and posterior maximum-intensity projection (MIP) views of a [^{18}F]fluoride PET scan showing numerous additional metastases. Even in retrospect, only a few of the [^{18}F] fluoride lesions can be seen on the [^{99m}Tc]MDP planar images. Figure adapted with permission of the Society of Nuclear Medicine from Bridges et al. [11, Figure 1].

bone cancers. Because ^{99m}Tc-labeled compounds have low toxicity, they can be used repeatedly in a subject without significant risk. One disadvantage of scintigraphy is that it cannot readily distinguish between benign and malignant disease. Scintigraphy differs in its sensitivity to detect metastastic tumors because bone formation and tumor growth rates differ in different tumors.

8.3.1.2 Computed Tomography

The first commercially available CT scanner was developed by Godfrey N. Hounsfield and announced in 1972. Allan McLeod Comack independently developed a similar scanner. In 1979, both shared a Nobel Prize in medicine.

In contrast to plain X-ray imaging, the X-ray beam in CT is collimated into a narrow beam that passes through the patient in thin slices from multiple angles. The beam is attenuated by its passage through different structures of the body and recorded by highly sensitive detectors capable of identifying slight differences in tissue density. The critical advance in the development and evolution of CT has been in the mathematics of the reconstruction algorithms that generate three-dimensional or tomographic images, and in the development of faster processing (computers) to perform the reconstructions. The scanner computes a tomographic data set that can be used for visualization of single or multiple slices in different planes (sagittal or coronal) [25]. CT images are obtained with specific contrast agents and provide both spatial and contrast resolution. This makes the images very useful for evaluating skeletal metastases. The detection of soft-tissue tumor extension by CT often depends on the difference in fat content of tumor and surrounding tissue. Conventional radiographic methods cannot detect tumor extension into bone marrow, but CT can. MRI however provides even better visualization of marrow invasion [98].

8.3.1.3 Magnetic Resonance Imaging (MRI)

The progress of MRI techniques in the course of the past two decades has had a major impact on imaging the musculoskeletal system. The contrast resolution of MRI is almost 50 times greater than that of conventional X-ray radiography and ~10 times greater than CT. Moreover, MRI does not expose patients to radiation [25]. MRI also makes it possible to perform magnetic resonance spectroscopy (MRS) and to identify the presence and concentration of specific, cancer-related molecules [47, 80]. MRI uses the magnetic properties of protons in the atomic nucleus to generate and detect signals that can be converted into an image or into a spectrum. Hydrogen-1 is the most abundant atom in human tissue and is responsible for the nuclear magnetic resonance (NMR) signal used to generate a traditional MRI. A key advantage of MRI is the number of image-aquisition sequences that can be used to create contrast within an image. The sensitivity of MRI permits bone tumors and invasion of bone marrow to be visualized. Using MRI soft-tissue tumor extension and "skip areas" of bone tumors can be readily detected. This is not true for other modalities. With MRI one can detect and distinguish residual tumor or recurrent tumor from nontumor-related abnormalities. Nevertheless MRI has limitations including low specificity: for example, infection, benign tumors, and trauma have often the same appearance as that of a malignant tumor. Nonetheless, MRI provides precise anatomic detail and excellent sensitivity for imaging bone marrow and facilitates the staging of known or suspected primary bone cancers [25].

8.3.1.4 Positron Emission Tomography (PET)

PET provides quantitative images of radioactivity in three-dimensional space, and is a widely used technique to trace images of physiological processes. PET was developed in the early 1970s, soon after CT and at about the same time as MRI. A major advantage of PET over gamma camera or SPECT imaging is the number of clinically useful short-lived positron-emitting radionuclides (e.g., fluorine ^{18}F, carbon ^{11}C, nitrogen ^{13}N, and oxygen ^{15}O) that can be used to label biologically relevant molecules such as glucose, amino acids, receptor ligands, etc. PET involves the emission of a positron from the nucleus, subsequent annihilation with an electron, and conversion into two 512 keV photons (gamma rays) emitted ~180^{O} apart. The simultaneous detection of these photons by the circular detectors of the tomograph provides the spatial

location of the positron emission, after reconstruction and correction for scatter and signal loss due to tissue attenuation. ^{18}F, ^{11}C, ^{13}N, and ^{15}O have been used extensively for PET imaging, with ^{18}F and ^{11}C the most widely used radionuclides to label molecules of biological interest.

The radiopharmaceutical that has had a major impact on clinical PET imaging is ^{18}F fluorodeoxyglucose (FDG), first synthesized in the mid-1970s. FDG is an analog of glucose that is transported across cell membranes and phosphorylated by hexokinase similar to glucose. Once phosphorylated, FDG-6-phosphate is not metabolized further, but becomes trapped inside the cell. The use of FDG to detect tumors is based on the studies by Warburg in the 1930s who showed that cancer cells have high rates of glycolysis [109]. Several features have contributed to the use of FDG in clinic. Unlike glucose, FDG is filtered in the kidneys and cleared from the circulation within 1 h after injection. Hexokinase activity is usually the rate-limiting step for both glucose and FDG utilization, and the enzyme is highly expressed in many tumors. The combination of high target tissue uptake and rapid blood clearance makes possible high-contrast images. In addition, the short 110-min half-life of ^{18}F permits FDG to be supplied from a central production facility. As a result FDG has found increasing use as an imaging agent [55, 96, 99, 110].

The application of PET in oncology began in the 1980s and has increased substantially since then [93]. [^{18}F]FDG-PET has been approved for diagnosing, staging, and restaging many cancers, including breast, lung, colorectal, lymphoma, melanoma, head, neck, and esophageal cancer. However, tumor cells are not the only cells with a high uptake of [^{18}F]FDG. Brain (grey matter) and non-fasting heart tissue have very high endogenous rates of glycolysis and correspondingly high rates of [^{18}F]FDG uptake. Recently, Maschauer et al. [73] have shown that endothelial cells in tumors and some vascular lesions exhibit high [^{18}F]FDG uptake and that [^{18}F]FDG uptake correlates with enhanced vascular endothelial growth factor (VEGF) expression. In addition, lesions with a high concentration of inflammatory cells, such as neutrophils and activated macrophages, also have increased [^{18}F]FDG uptake. This is important because inflammation may be mistaken for malignancy in patients with proven or suspected cancer [12].

The use of ^{18}F-fluoride for bone scintigraphy dates back to the early days of bone imaging in the 1950s and early 1960s, but the production of this isotope requires the presence of a nearby cyclotron [100]. In 2000, as part of the modernization of the Food and Drug Administration and the improved handling of new drug applications, ^{18}F-fluoride, ^{13}N-ammonia, and [^{18}F]FDG were approved for use in PET for bone scintigraphy, cardiac perfusion, and oncology/neurology. ^{18}F bone scintigraphy has been applied to bone scanning [58, 77], but ^{18}F PET bone imaging is more sensitive and has greater specificity [97]. ^{18}F PET bone scans are easy to perform and readily adaptable to general clinical imaging (Fig. 8.5) [11]. Similar to CT and MR equipment, PET instrumentation has improved dramatically over the past two decades. The system resolution for patient imaging is now 4–5 mm, significantly greater than the 15-mm resolution of the initial systems. Furthermore, a multimodality approach to imaging patients has developed recently. By combining CT scanners with PET scanners, PET images are of better quality and the combined devices provide co-registration and shorter imaging times (Fig. 8.6). The development of combined MRI/PET scanners for clinical use is rapidly proceeding and they are likely to become the norm in the next 5 years.

8.3.2 The Applications of Imaging Technologies in Mouse Models

Since the 1980's, mouse genetic modification techniques have been used to develop new mouse strains. Transgenic mice are now commonly used to study different diseases, including cancer. However, no adequate animal model exists for the study of cancers with bone involvement (see Chapter 12 in this volume). The most common method to develop an animal model of osteosarcoma, one of the most studied bone cancers, is to inject cells orthotopically, directly into bone, or by intravenous administration [28, 27]. Models of bone metastasis have

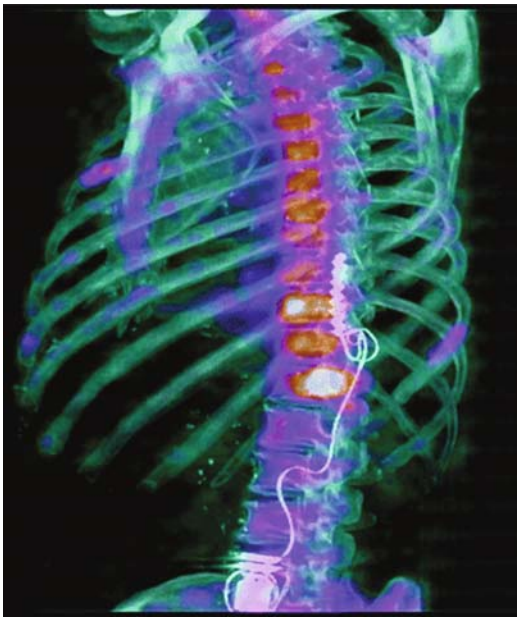

Figure 8.6. Three-dimensional [^{18}F] fluoride bone scan PET/CT fused image. Fused images show rib and vertebral metastases. Electronic pain control module and electrodes are also visualized on the CT. Figure adapted with permission of the Society of Nuclear Medicine from Bridges et al. [11, Figure 3].

used other approaches [111], for example, injection of tumor cells into the left ventricle of the heart [92]. However, this model does not fully reflect the metastatic process, that is, tissue invasion at the site of the primary tumor, tumor cell entry into the lymphatics and vasculature, localization in and exit from the bone vasculature, followed by proliferation in a bone marrow microenvironment where bone structure and function have been modified. Bone metastasis models that have been generated by the methodologies referred to above [92, 111] can be defined as "end-stage" lesions with highly proliferating cells and modifications of bone structure that are secondary to tumor growth. Bone lesions resulting from these methods can be identified by X-ray radiography and random histopathology [121], but the techniques are insufficiently sensitive, with the result that the actual number of bone metastases is likely to be underestimated. Radiography detects severe lesions and does not reveal bone metastases. Moreover radiography will not detect bone metastases that fail to induce severe bone lysis or induce formation of a mineralized matrix. For this reason it has been necessary to apply other imaging modalities in patient care. Recently these modalities have also been utilized in mouse models for the study of tumor cell growth in bone.

The application of the most widely used molecular imaging modalities to study bone cancers in mouse models will now be reviewed. These include optical (fluorescence, bioluminescence) imaging, and the methods generally used in patient care, that is, PET, SPECT and CT.

8.3.2.1 Bioluminescence and Fluorescence Imaging in the Detection of Bone Metastasis Coupled with Other Imaging Techniques

The application of the two most widely used reporter systems – Firefly luciferase (FLuc) and green fluorescence protein (GFP) – dramatically improved the imaging of bone metastases with currently available optical instrumentation. Once reporter gene(s) have been introduced into cells and cells bearing the reporter system have been selected, it becomes possible to track the cancer cells that stably express reporter systems. This is more sensitive and faster than the traditional histopathological or immunohistochemical procedures. In particular, GFP labeling

has significantly improved the ability to visualize metastases in fresh soft-tissue organs and bone. For example, injection of B16F0-GFP mouse melanoma cells into the vein or portal vein of 6-week-old C57BL/6 and nude mice led to metastatic lesions in the brain, liver, and bone. This was revealed by whole-body optical imaging, performed with either a transilluminated epifluorescence microscope or by a fluorescence light box and a thermoelectrically cooled charge-coupled device camera. The depth to which metastasis and micrometastasis are imaged depends on their size. A 60-μm diameter tumor can be detected at a depth of only 0.5 mm, whereas a 1,800-μm tumor can be visualized at 2.2-mm depth [9, 120]. More detailed work has utilized the same approach [86] on a different cell line (MDA-MB-435, human breast cancer cells) that also expressed GFP.

Research has focused on several metastatic events. At the site of metastases, tumor cell arrival, localization, and initial colonization have been studied following the injection of MDA-MB-435 cells into the cardiac left ventricle of athymic mice. Femurs were analyzed by fluorescence microscopy, immunohistochemistry, real-time PCR, flow cytometry, and histomorphometry at periods ranging from 1 h to 6 weeks after intracardiac injection. Single cells were found in distal metaphyses at 1 h post-injection and remained as single cells up to 72 h. Diaphyseal arrest occurred rarely and few cells remained after 24 h. At 1 week, numerous foci (2–10 cells) were observed, mostly adjacent to osteoblast-like cells. By 2 weeks, fewer but larger foci ($\geq$50 cells) were seen. Most bones had a single large mass at 4 weeks (originating from a colony or coalescing foci) which extended into the diaphysis by 4–6 weeks. Little change ($<$20%) in osteoblast or osteoclast numbers was observed at 2 weeks; but, at 4–6 weeks osteoblasts were dramatically reduced (8% of control), while osteoclasts were reduced only modestly (to ˜60% of control). With the aid of this GFP model of how breast cancer metastasizes to bone, it became possible to try to identify key tumor cell–bone–cell interactions and when they occurred during bone metastasis (Fig. 8.7).

BLI has been used mainly to detect primary xenografts growth and metastasis in animal models [21, 20, 30]. The MDA-MB-231 breast cancer cell line is widely studied because it can form metastases after intracardiac injection. With the aid of firefly luciferase-expressing subclones, D3H1 and D3H2LN, MDA-MB-231 cells have been shown to produce multiple metastases at high frequencies in tissues that mimic the human disease, such as bone and brain. These subclones also produce lymph node and lung metastases. Jenkins et al. [65] have demonstrated that luciferase expression is stable even after multiple rounds of in vivo growth or continuous culture. In another study, the increased sensitivity due to BLI coupled with fluorescence microscopy allowed characterizing the metastatic activity of individual single-cell progenies (SCPs) by evaluating their tissue tropism and growth kinetics (78, Fig. 8.8). Some SCPs were found to metastasize to bone, while others metastasized better to lung, and a minority was also able to colonize and grow within the adrenal gland. This organ-specific metastatic pattern resembles the typical distribution of breast cancer metastases observed in patients [78]. Transcriptomic profiling revealed that the more metastatic of these different SCPs express a previously described “poor-prognosis” gene expression signature [67].

Conventional imaging techniques, including X-rays, MRI, SPECT, or PET, in mouse models are limited with respect to imaging tumor growth in bone. Radiography is not sufficiently sensitive to detect early bone metastases. Microcomputerized tomography is much more sensitive, but is not widely available. Except in small studies, serial sectioning (required to locate rare single cells) becomes time- and cost-prohibitive. As a result, experiments have been largely limited to the study of late events in metastatic bone disease, such as osteolysis, but earlier events such as arrival, lodging, colonization, and intraosseous trafficking of tumor cells have not been adequately studied.

Progress has been made in the use of high-resolution microCT to image and quantify morphometric and structural changes in bone. There is advantage to combining microCT and in vivo BLI in experimental murine models to

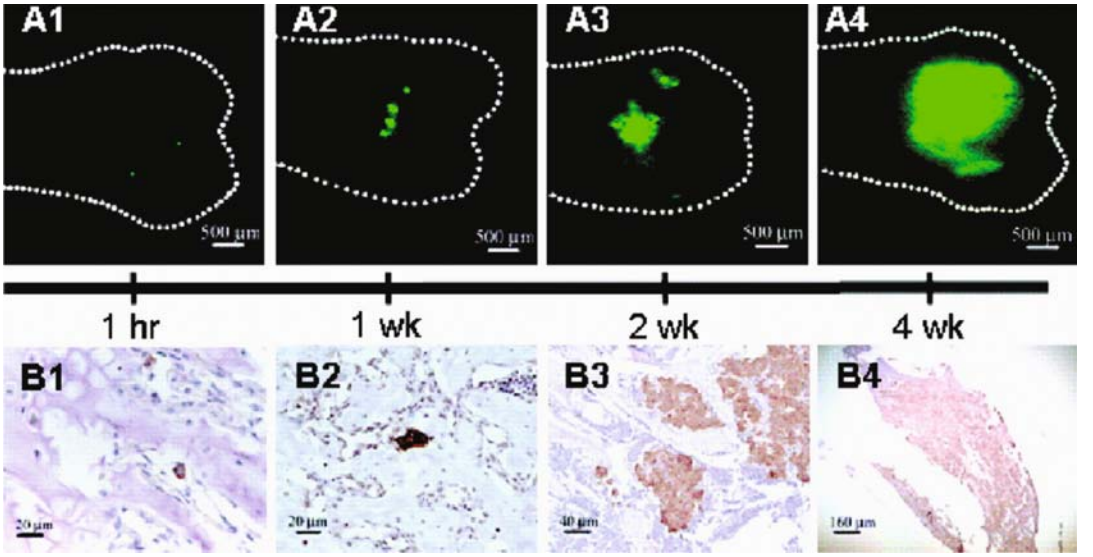

Figure 8.7. The kinetics of MDA-435GFP metastatic growth in the femur following intracardiac injection. Whole femurs were dissected and fluorescent foci were visualized in the intact bones using a fluorescence stereomicroscope. (**A)** Fluorescent foci were initially observed mainly in the distal end of femurs (1 h after injection **A1**, 1 week **A2**, 2 weeks **A3**; and at 4 weeks, **A4**). (**B)** MDA-435GFP cells were detected by anti-GFP immunohistochemistry (*brown* staining cells) in femurs at 1 h (**B1**, single cell), at 1 week (**B2**, clusters of 2–3 cells), at 2 weeks (**B3**), and at 4 weeks (**B4**). Figure adapted with permission from Phadke et al. [86].

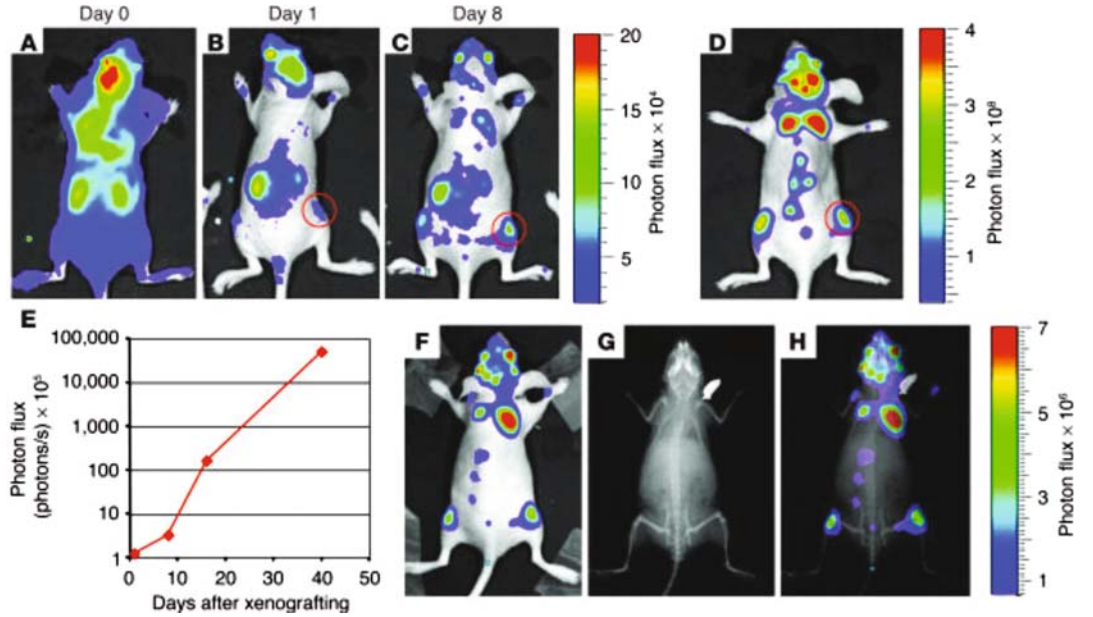

Figure 8.8. Noninvasive bioluminescence imaging to monitor the development of osteolytic metastases from the same mouse. (**A**–**D**) SCP2, a highly metastatic clone from MDA-MB-231, was transduced with the luciferase-containing TGL reporter gene and injected into the left cardiac ventricle of an immunodeficient mouse. The bioluminescence signal was captured at the indicated times after xenografting. The intensity of the signal, measured as photon flux, is shown as a color scale. Images for days 0, 1, and 8 are displayed on the same scale, while the day-35 image is shown on a different scale, due to the exponential growth of the metastases. A metastasis to the *right* hindlimb is circled in *red*. (**E**) The growth kinetics of the *right* hindlimb metastasis outlined by the *red* circle shown in **B**–**D** was quantified by measurement of photon flux. (**F**–**H**) A bioluminescence image (**F**) and a skeletal X-ray image (**G**) were obtained on day 16 after xenografting. Images were superimposed (**H**) to demonstrate registration of the bioluminescence signals with skeletal anatomy. Figure adapted from Minn et al. [78]. Copyright 2005 by the American Society for Clinical Investigation. Reproduced with permission of the American Society for Clinical Investigation via Copyright Clearance Center [78].

study the kinetics of intraosseous prostate cancer (CaP) growth and the appearance of bone lesions [42]. To mimic osteolytic bone metastasis, the left tibias of SCID mice were injected with the human CaP cell line PC-3 expressing luciferase (PC-3 Luc). Noninvasive monitoring of tumor progression was followed by weekly BLI for a period of 4 weeks. Bone morphometric parameters were quantified by microCT. BLI monitoring in vivo revealed a PC-3 Luc tumor as early as 7 days and exponential tumor growth after 2 weeks, but a decrease of bone density and bone content was established by microCT as early as 7 days post-injection and reached significance at day 21 (30% and 25% loss, respectively, compared with controls). Enhanced osteoclast TRAP activity was observed during the first two weeks. This highlights early interaction between low proliferative PC-3 cells and osteoclasts when the tumor becomes established in bone. Tumor growth assessed by BLI was closely correlated with osteolysis, as assessed by microCT ($p<0.05$). These results show that microCT and BLI can be successfully combined to evaluate kinetics of intraosseous tumor growth and bone destruction [42].

In a study of a metastatic melanoma model [32], PET and BLI technology with CT were combined to obtain fusion images that provide both molecular and anatomic information on metastases subsequent to intracardiac injection of a melanoma cell line (A375M-3F) transduced with a lentiviral vector that contained a trimodal imaging reporter gene. The gene was encoded with a fusion protein including *Renilla* luciferase, monomeric red fluorescent protein, and a mutant herpes simplex virus type 1 thymidine kinase. Co-registration of the PET and CT images provided multimodal imaging that confirmed the metastatic sites in the mouse xenograft models (Fig. 8.9) [42].

Concerns have been expressed whether increasing utilization of CT and SPECT and repetitive X-ray exposure from CT and radionuclide imaging affect the cancer cells and the metastatic process itself, that is, whether the radiation exposure introduces an additional variable into the experimental design [24]. A histomorphometric study indicated that weekly exposure to CT X-rays over a 5-week period slightly increased the number of metastases, specifically in the distal femur and proximal tibia of nude mice. These findings suggest that imaging modalities utilizing X-rays in animal models can influence cancer metastasis and should be used with caution and moderation. The sensitivity of BLI is high, but specificity, spatial accuracy, and predictive value have been shown to vary and depend on the location of the metastases. Even though BLI assessment of total body and bone metastases is not as sensitive or as rigorous as whole-body histomorphometry, it is more rapid and cost-effective. MicroSPECT/CT imaging in nude mice using ^{99m}Tc-labeled diphosphonate has been disappointing and cannot identify bone metastases in the MDA-MB-435 breast cancer cell line [24].

[^{18}F] fluoride ion bone scans in mice have been used to monitor benign and malignant changes in the mouse skeleton by microPET [5]. These studies showed that traumatic changes in the murine skeleton were easily detected by this technique. Malignant bone tumors induced by the injection of prostate cancer cells into the tibia were detected by X-ray and histology and compared to the images obtained by [^{18}F] fluoride PET. Osteoblastic behavior of LAPC-9 tibial tumors, indicating very high bone turnover, was readily demonstrated by the high uptake of [^{18}F] fluoride on microPET scans, with osteoblastic lesions ˜2 mm diameter clearly visible. However, the trauma associated with the injection procedure has called into question the specificity of microPET images of tumor induction. Osteolytic tumors sometimes showed high bone turnover at the lesion edges, but in most cases presented as [^{18}F] fluoride "cold spot" in the functional scans and were therefore more difficult to detect with microPET. This work provides a foundation for quantitative, high-resolution functional bone imaging in mice [5].

MRI, because of its high resolution and ability to produce tomographic images of entire organ systems with exquisite soft-tissue contrast without the use of ionizing radiation is commonly employed to track tumor size, location, and metastatic burden [113]. MRI contrast agents, such as superparamagnetic iron oxide (SPIO) nanoparticles and micron-sized iron oxide particles (MPIO) [54], have been used to tag and detect cell populations in animals and humans.

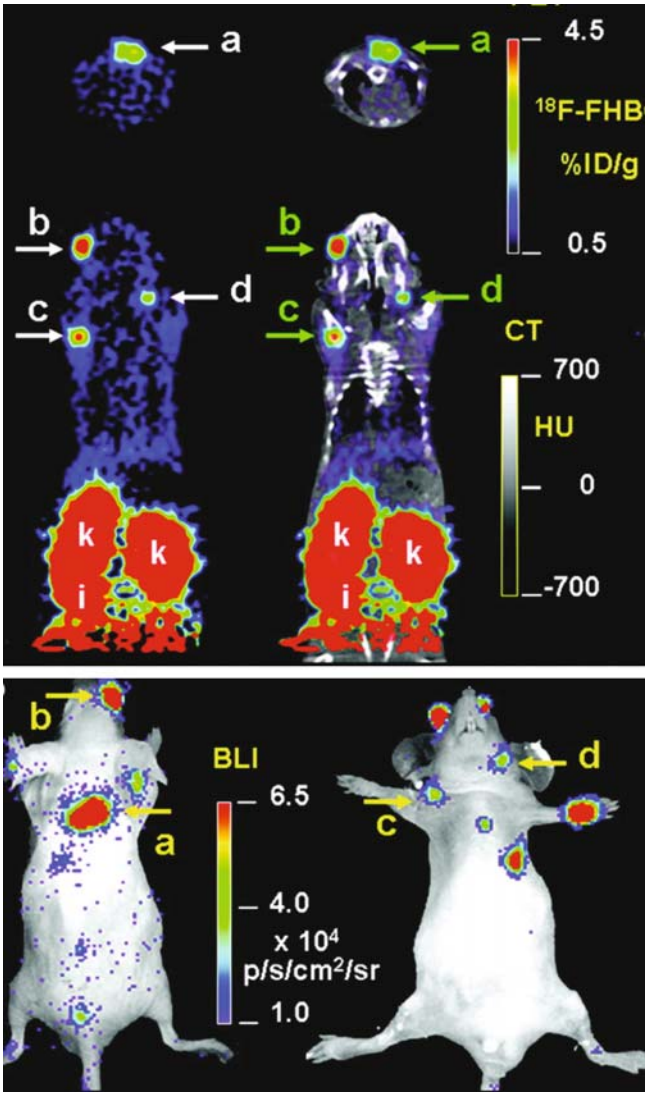

Figure 8.9. The value of CT in [^{18}F]-FHBG PET images of metastasis. (**A**) Thirty-five days after intraventricular injection of 1.5×10^6 A375M-3F melanoma cells in nude mouse, [^{18}F]FHBG PET/CT allows precise anatomic localization of metastasis to the interscapular fat (a), right eye (b), right humeral head (c), and left mandibula (d) as shown by *green arrows*. Lack of anatomic landmarks on PET alone is illustrated by *white arrows*. (**B**) BLI shows same lesions as seen on [^{18}F]FHBG PET/CT, but does not provide information on depth of the lesion. Figure adapted with permission of the Society of Nuclear Medicine from Deroose et al. [32, Figure 6].

A multimodality approach, for example, a combination of MRI and BLI, has been used to assess bone precursor cell (BPC) biology in living animals. Combined imaging technologies have been used for noninvasive monitoring of BPCs within the marrow space of murine long bones [75]. 7F2 cells and preosteoblast MC3T3-E1#4 cells (committed BPCs) were labeled with MPIOs and imaged inside the bone marrow cavity of living mice. Because these cells were stably transduced with a triple-fusion imaging reporter [89] using a retrovirus, it was possible to monitor dual MPIO-reporter gene labeled by sequential MRI–BLI imaging which tracked the time course of organ distribution and gene expression [5]. This multimodal approach may

have advantages, especially for the tracking of breast cancer cells in mouse models of brain and/or bone metastasis. Up to now these were detected only by magnetic resonance MPIO imaging (MRI) [53].

8.4 Issues for the Future

Noninvasive reporter gene imaging is an exciting, indirect imaging strategy that can be fully exploited in experimental and transgenic animals. Direct imaging probes have advantages and will continue to be developed and to make significant contributions to molecular imaging. Direct imaging probes have the major advantage that they do not require the transduction of target tissue by a reporter gene-bearing vector.

Two areas in bone cancer research require future development: (1) appropriate animal models, and (2) multimodality imaging strategies. The development of appropriate animal models that allow investigation of the early stages of the bone metastatic process is critical. Investigators are seeking better identification and characterization of the cancer cell "niche" within bone (see Chapter 1 in this volume). The factors that maintain cancer cell dormancy within the bone microenvironment are also critical, as are the factors that initiate transition from cancer cell dormancy to lesion proliferation leading to tumor growth and disease progression in bone.

To achieve these objectives, multimodality imaging strategies, ranging from in vivo noninvasive macroscopic imaging to in situ minimally invasive (optimal window) microscopic imaging can provide the appropriate tools. The transition from macroscopic to microscopic imaging is likely to be crucial. Macroscopic imaging of small cell clusters in bone and bone marrow is a challenge, even in small rodent animal models. Constitutive and inducible reporter-gene imaging for tracking and monitoring signaling-pathway activity, respectively, can provide unique information in single animals followed serially over time. Bioluminescence imaging with luciferase-transduced tumor (tumor-stem) cells is limited to ~10–100 cells, whereas high-resolution in situ fluorescence imaging can detect individual cells, but requires special optical windows and provides only a small field of view. Nonetheless, the combination of new animal models and reporter-transduced cancer (cancer-stem) cell lines with a multimodality approach to imaging macroscopic and microscopic disease will provide the best approach to the study of bone metastases in the near future.

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9.

Inflammatory Cytokines and Their Role in Bone Metastasis and Osteolysis

Larry J. Suva, Richard W. Nicholas, and Dana Gaddy

9.1 Introduction

Cancer can be promoted and/or exacerbated by a number of infectious and inflammatory processes [72]. Indeed, inflammation produces a supportive tumor microenvironment that is likely a vital component of the metastatic cascade. The mechanisms that link inflammatory responses with cancer development and metastasis are the cytokines and chemokines that are produced by activated innate immune cells or the tumor cells themselves, stimulating and supporting tumor growth and progression. Some soluble tumor-derived mediators can recruit and activate local inflammatory cells, thereby further stimulating tumor progression. This chapter provides an overview of the role of inflammatory cytokines and chemokines in tumor progression and osteolytic bone metastasis.

9.2 The Link Between Inflammation and Cancer Progression

Cancer is a disorder of exuberant cell proliferation. It involves a poorly understood sequence of molecular events that include morphological and cellular transformation, dysregulation of apoptosis, uncontrolled cellular proliferation, invasion, angiogenesis, and metastasis [72], with chronic infection, inflammation, and cancer closely associated [3, 26, 79]. Inflammation was first linked with cancer when it was shown clinically that infiltration of innate immune cells into malignant tissue was associated with poor clinical outcome [26]. Subsequent population-based studies demonstrated an increased cancer incidence in patients affected by chronic inflammatory disorders such as pancreatitis, Crohn's disease, and ulcerative colitis [107, 117]. It has become abundantly clear that the tumor microenvironment, which is predominantly managed by inflammatory cells, is an indispensable participant in tumor development [26, 28]. The host microenvironment facilitates and supports tumor proliferation, survival, migration, and homing. In addition, it now appears that tumor cells have co-opted many of the signaling molecules of the innate immune system, including selectins [58], chemokines, and their receptors [22, 89], as well as the NFκ-B signaling pathway, which itself is activated by many pro-inflammatory cytokines [21, 103]. It is thus apparent that an inflammatory microenvironment supports tumor progression, invasion of surrounding tissues, angiogenesis, and metastasis [72].

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Metastasis, the spread of cancer cells from primary tumor sites to distant organs and tissues, accounts for over 90% of lethality in cancer patients [11], yet the underlying cellular and molecular mechanisms of metastasis remain poorly understood.

9.3 The Inflammatory Bone Microenvironment in Cancer Progression

Bone is a common site for metastasis. These bone metastases are frequently associated with intractable pain, pathological fractures, nerve compression, and hypercalcemia due to osteolysis [88]. In addition, for skeletal tumors such as multiple myeloma or the bone metastases of breast and prostate cancer, the associated extensive bone destruction often denotes a dramatic worsening in the prognosis for the patient [17]. Much of the work on metastasis to bone has focused on the respective roles of tumor cells, osteoblasts and osteoclasts. Furthermore, the bone metastasis microenvironment involves the presence and activity of many cell types in addition to those normally resident in bone tissue.

Metastasis is a non-random process [45]. Some tumors prefer specific organs to which to metastasize. Other tumors are less selective. Site selectivity depends on tumor cells being able to carry out all the steps of the metastatic cascade [34, 35]. Even though anatomical and mechanical factors such as blood flow affect the sites of tumor metastasis, it is ultimately the microenvironmental factors that determine the preferred metastatic site(s) and promote tumor proliferation. This is accomplished by manipulating the host microenvironment by processes such as the secretion of systemic factors from the primary tumor site [56], by inducing tumor cells to adhere to distant endothelial cells by extravasation into the target tissue, and by colonizing the target to bring about growth of the lesion [11].

The microenvironment surrounding tumor plays a critical role, at least equal to that of tumor cells themselves, in the progression of cancer [26, 77]. This role also extends to the extracellular matrix. The microenvironment consists of stromal fibroblasts, components of the vasculature, normal epithelia, extracellular matrix, and inflammatory cells, and interacts with tumor cells through the release of growth factors, cytokines, proteases, and other bioactive molecules. Tumors often arise at sites of injury or infection where immune cells congregate, secrete growth factors to promote healing, and cause reconstruction of the extracellular matrix [104, 72].

The cellular milieu of the inflammatory tumor microenvironment consists of resident and recruited macrophages [69], dendritic cells (DCs), T cells, natural killer (NK) cells [72] (Fig. 9.1), and leukocytes that are prominent in primary and metastatic tumors [8]. Infiltrated immune cells can exert paradoxical effects during cancer progression and may even regulate bone remodeling [36]. Yet, the means by which host immune cells contribute to the microenvironment of osteolytic bone metastases has received little attention. The more recent and welcome use of immune-replete animal models may fill this obvious void.

9.4 Mediators of the Inflammatory Response

When immune cells and developing tumor cells localize to a common microenvironment, the tumor may be abolished as a result of immunosurveillance or it may survive and progress. Inflammatory cells in the tumor–host microenvironment, such as lymphocytes, mast cells, neutrophils, and macrophages, secrete cytokines, growth factors, and chemokines that affect survival (Fig. 9.1). It was endogenous cytokines that were thought to modulate host responses [72], particularly of inflammatory cytokines that support or retard tumor progression [4, 72].

This chapter focuses on the role of a select group of cytokines produced by cells within the microenvironment of bone metastases on tumor progression within bone. These are tumor necrosis factor-α (TNF-α), interleukins (IL)-6, IL-8, *IL-10 (inhibitory)*, IL-12, IL-23, CXCL12/CXCR4, and transforming growth factor-β (TGF-β) (Fig. 9.2). Each cytokine was chosen because it has a specific function in

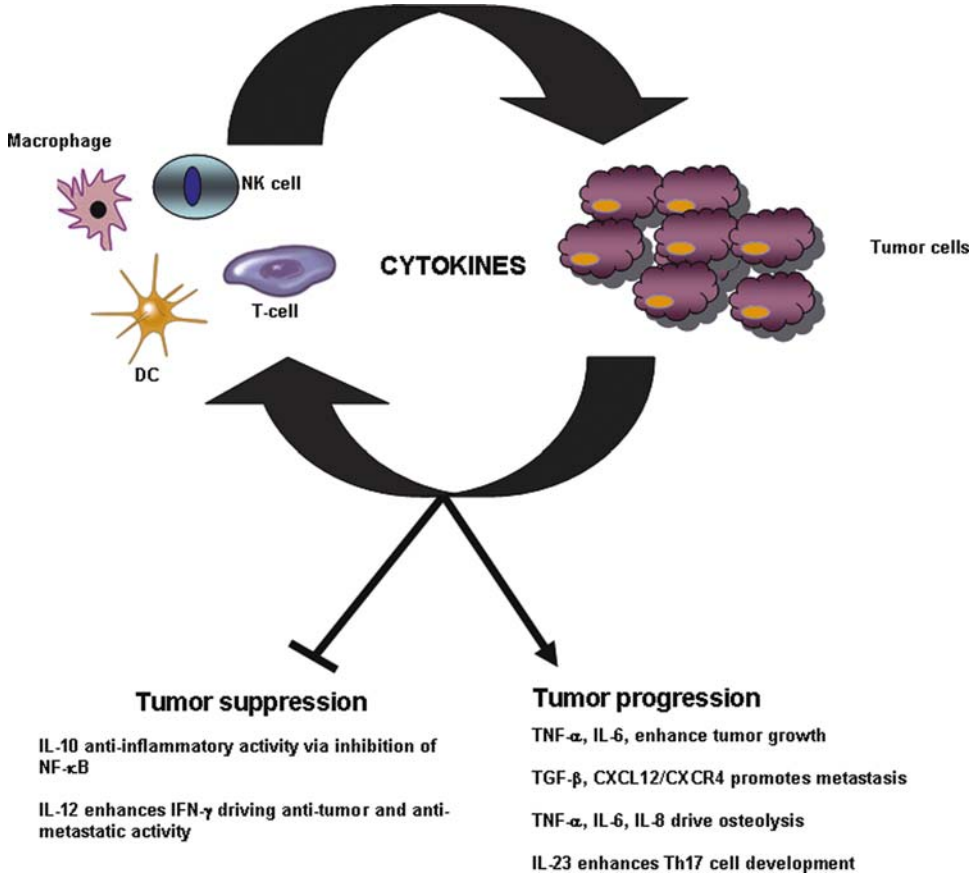

Figure 9.1. The potential interactions between tumor cells and infiltrating inflammatory and/or immune cells in the tumor microenvironment. Cytokines secreted by tumor or inflammatory/immune cells either promote tumor development and survival or exert potent anti-tumor effects. The chemokine CXCL12 and its cognate receptor CXCR4 support metastasis and tumor progression. Substantial inflammation develops through the action of various inflammatory mediators, including TNF-α, IL-6, IL-8, and IL-23, leading to loss of anti-tumor immunity, bone resorption, and substantial tumor progression. However, IL-10, through anti-inflammatory effects, and IL-12, through production of IFN-γ and activation of T and NK cells, lead to tumor suppression. The multiple actions of TGF-β (tumor-derived and from bone resorption) and IL-23 (via activation of IL-17) explain their dual roles in tumor development and progression of bone metastases.

modifying the bone microenvironment and thus altering tumor progression. For additional information regarding the roles of other cytokines in tumor progression, the reader is directed to some of the many reviews of inflammatory cytokine action [4, 22, 36, 72].

9.5 TNF-α

The critical role of TNF-α in chronic inflammatory diseases is well established [72] and its role as an anticancer cytokine has been known for the past two decades [2]. However, TNF-α also can stimulate tumor progression by mediating the proliferation, invasion, and metastasis of tumor cells [1].

TNF-α, produced by a wide variety of tumor and inflammatory cells, supports tumor cell progression and survival via the induction of NF-κB-dependent signaling pathways. These in turn cause induction of anti-apoptotic molecules [76] and activation of osteoclastogenesis [59]. Some genetic polymorphisms that result in increased TNF-α production have been associated with an increased risk of multiple myeloma [5, 54], breast cancer [40], bladder cancer [80], and correlate with poor prognosis in other

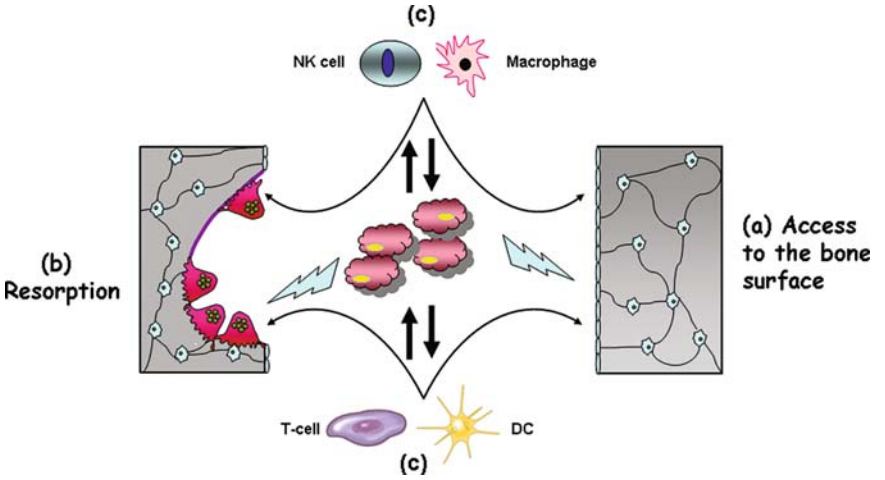

Figure 9.2. The tumor microenvironment includes interactions between tumor cells, infiltrating inflammatory and/or immune cells; natural killer (NK) cells, macrophages, T-cells, dendritic cells (DCs) and cells resident in bone (osteoclasts, osteoblasts, lining cells, and osteocytes). Cytokines secreted by tumor cells drive tumor development and survival by activating bone cell motility; allowing access of tumor products (and perhaps tumor cells) to the bone surface (**a**) and/or directly activating osteoclast differentiation and activity (**b**). In addition, the tumor-derived cytokines also stimulate responses in the resident inflammatory/immune cells in the tumor microenvironment (**c**). These interactions can then modify tumor cell function and/or contribute to the activation of resident bone cells. In sum, tumor cells integrate the microenvironmental signals from the activity of immune cells, bone cells, and the tumor cells themselves, resulting in the increase in bone resorption that is characteristic of osteolytic bone metastasis.

hematological malignancies [85]. TNF-α confers an invasive, transformed phenotype on mammary epithelial cells [86].

The loss of cell–cell adhesion and the resulting increase in the ability of tumor cells to invade are crucial for metastasis [12], yet the molecular mechanisms that underlie these processes are not fully understood. In Ewing's sarcoma TNF-α induces bone resorption [68] and, in malignant glioma, it upregulates angiogenic factors [91], thereby promoting angiogenesis and tumor progression. This induces upregulation of TNF-α, IL-8, and, to a lesser extent, vascular endothelial growth factor (VEGF) [91]. Upregulation of TNF-α also impairs immune surveillance because T-cell responses and the cytotoxic activity of activated macrophages are strongly suppressed [32].

The increase in bone resorption due to TNF-α can be the result of direct action by TNF-α [21], of increased angiogenesis or due to IL-8, a molecule that directly activates osteoclastogenesis independent of RANK ligand (RANKL) [13]. Collectively, the increase in TNF-α expression by tumor and inflammatory cells mediates a myriad of cellular effects that ultimately lead to increased osteolysis and tumor progression. However, TNF-α is far from being the only cytokine expressed in the bone marrow microenvironment.

9.6 TGF-β

TGF-β is one of more than 30 peptide growth factors with pleiotropic effects in a variety of tissues. TGF-β signaling is initiated by ligand binding to the type II (TβRII) receptor serine kinase on the cell surface [109]. This interaction allows TβRII to phosphorylate the type I receptor (TβRI), thereby propagating signal transduction via phosphorylation of specific downstream regulatory Smads, Smad2 and Smad3 [109]. Smad2/3 binding to the common Smad4 leads to nuclear translocation of the complex and to direct or indirect DNA binding that stimulates target gene transcription and changes in cell phenotype [109].

TGF-β regulates a broad range of cellular functions, including proliferation, cell differentiation, immune surveillance, extracellular

matrix secretion, cell adhesion, apoptosis, and even specification of developmental cell fate [82, 100]. The role of TGF-β in mediating the progression of osteolytic bone metastasis is well-established [44]. Rather, the diverse and often ignored role of TGF-β in the regulation of tumorigenesis and cancer progression will be focused on.

As with many cytokines, TGF-β is a powerful pleiotropic immunosuppressive and anti-inflammatory agent. It also regulates T-cell proliferation and function [72], as exemplified in studies with TGF-β knockout mice [63]. These mice suffer from lethal multifocal inflammatory disease; that points to the importance of TGF-β in maintaining immune system homeostasis [116]. Similarly, the blockade of TGF-β signaling in T cells via transfection with dominant-negative TβRII [41] or by conditional deletion of TβRII in bone marrow, led to the same multifocal inflammatory responses [70].

TGF-β is also a key player in malignant disease through its effects on tumor cells, non-transformed cells present in the primary tumor, distal host cells that result in suppression of anti-tumor immune responses, and distant cells at potential metastatic sites [116]. The increased TGF-β secretion associated with tumor masses and the normal levels associated with cells of the immune system contribute to the high TGF-β levels observed in cancer patients.

A high TGF-β level suppresses innate anti-tumor immune responses and supports angiogenesis, invasion, metastasis, and the increase in tumor extracellular matrix deposition which ultimately bring about tumor progression [45, 116].

TGF-β is also released from the extracellular matrix of bone via osteoclastic bone resorption [27]. This bone-derived TGF-β further stimulates bone cell and tumor cell proliferation [43]. TGF-β is also secreted by bone marrow mesenchymal cells, resident leukocytes, monocytes, and macrophages recruited to the tumor, all of which contribute to innate immune suppression [116].

Collectively, the increased TGF-β secretion by a variety of tumor cell types and by cells in the local tumor environment results in elevated plasma levels of TGF-β. These in turn are associated with advanced-stage disease, i.e., the presence of metastases and poor patient outcome. TGF-β levels therefore may become a prognostic marker for advanced breast cancer [49], prostate cancer [126], and pancreatic cancer [37]. TGF-β levels are also elevated in renal cell carcinoma [51] and may be the cause of local immunosuppression. Elevated serum levels of TGF-β in multiple myeloma patients are equally likely to be an adverse prognostic marker of the disease [121]. In multiple myeloma the largest source of TGF-β are malignant myeloma cells, with a smaller component derived from bone marrow stromal cells [120].

TGF-β and components of the TGF-β signaling pathway exert pleiotropic effects on many steps of the metastatic cascade. They stimulate angiogenesis, activate the epithelial–mesenchymal transition, suppress immune surveillance, stimulate tumor invasion, and increase tumor extracellular matrix production [42]. Activated Smad signaling has been demonstrated in breast cancer cells resident in the bone microenvironment [52]. Diminished TGF-β signaling in animal models of both breast cancer and melanoma resulted in dramatically decreased bone metastases [50, 129]. Small-molecule inhibitors of the TβRI kinase have been developed that suppress the in vitro invasion and in vivo bone metastases of breast cancer cells in murine models [9, 31].

The importance of TGF-β in regulating tumor progression is brought out in tumors with defective TGF-β signaling. In colon cancer, the mutational inactivation of TβRII [97] inhibits potently the growth of colon epithelial cells and the T-cell-mediated tumor suppressive activity [30].

Targeting TGF-β signaling pathways shows promise for the treatment of metastatic cancer, either via direct effects on the tumor cells themselves, or via immuno-modulation of the cellular milieu within the tumor microenvironment. The potent immunomodulatory effects of TGF-β highlight the importance of utilizing immune competent animal models in preclinical studies with anti-TGF-β agents. Clinical trials with small-molecule inhibitors have been initiated.

9.7 Interleukin-6

IL-6 is a potent pleiotropic inflammatory cytokine that is a key growth-promoting and anti-apoptotic factor [48], as well as a well-characterized pro-osteoclastogenic factor associated with osteoporosis and hypercalcemia [78, 92]. The IL-6 receptor complex is a heterodimer that consists of IL-6Rα and glycoprotein 130 (gp130), with the latter responsible for signal transduction [78]. Most IL-6 target genes are associated with cell cycle progression and the suppression of apoptosis. IL-6 also plays an important role in the pathogenesis of multiple myeloma [19, 128] and increased IL-6 expression is correlated with an increased risk of developing breast cancer [47]. A G/C polymorphism within the promoter region of the IL-6 gene is associated with breast cancer survival. This points to a mechanistic link between host environment and tumor growth [16]. Furthermore, five IL-6 single-nucleotide polymorphisms (SNPs) and IL-6 haplotypes based on these SNPs are associated with an increased risk of breast cancer [112].

IL-6 acts as a paracrine growth factor for multiple myeloma, non-Hodgkin's lymphoma, bladder cancer, colorectal cancer, and renal cell carcinoma [72]. In the case of multiple myeloma (see Chapter 3), the increase in cytokines, chemokines, and cell–cell contacts provided by the bone marrow microenvironment supports multiple myeloma cell survival and proliferation [127].

The interaction of the receptor activator of NF-κB (RANK), located on osteoclast precursors, with RANKL on osteoblasts and bone marrow stromal cells, is driven by IL-6 and other cytokines such as MIP-1α, IL-1, PTHrP, and IL-8, secreted by multiple myeloma cells (see Chapter 3). It is this interaction that mediates bone destruction in multiple myeloma [101]. It is likely that the increase in bone resorption also is elevated in metastatic breast and prostate cancer [11, 75] and is the result of paracrine loops between tumor cells, immune cells, and osteoclasts.

9.8 CXCL12/CXCR4

Chemokines and their receptors play an important role in the organ to which particular cancer cells metastasize. Chemokines are small (8–10 kDa), inducible, secreted chemoattractant cytokines (CXC- or α-chemokines) that bind to G-protein-coupled seven-span transmembrane receptors. They are secreted by virtually all types of cells, but the amounts and their combinations are often modulated by inflammatory cytokines, growth factors, and tumor cells [4, 7, 89]. When chemokines are bound to their receptors, many downstream signaling pathways are activated. This includes non-receptor tyrosine kinases, MAPK, or protein kinase C [123]. Chemokines are classified structurally into four separate groups, depending on the number and position of conserved cysteines. CXC (α-chemokines), CC (β-chemokines), and CX3C chemokines have four conserved cysteines, with either no, one, or three amino acids separating the first two cysteines [123].

In addition to their primary and physiologically critical function in regulating leukocyte trafficking, chemokines also have key roles in embryogenesis, organogenesis, and tissue homeostasis. In addition, they, along with their receptors, are now known to be involved in inducing tumor cell migration [123], in regulating tumor growth, angiogenesis, invasion, osteolysis, and metastasis of the transformed phenotype [11, 89].

Perhaps the best studied example is the CXC chemokine CXCL12 and its cognate receptor CXCR4 in metastatic breast cancer [87]. Other chemokine/receptor pairs such as CCR7/CCL21 and CCR10/CCL27 [10] and the CCR5/CCL5 axis [53] also support metastasis formation at preferred sites.

The ability of a specific chemokine to act on chemokine receptor-expressing tumor cells requires that the chemokine-induced cellular changes culminate in cellular motility in direct response to specific chemokine gradients [87], resulting from a highly potent axis between CXCL12 and its CXCR4 receptor [87].

CXCL12 is expressed constitutively in breast cancer metastatic sites, such as bone marrow, lung, liver, and lymph nodes [87]. In addition, CXCL12 induces the homing of hematopoietic stem cells to the bone marrow via CXCR4 [67]. This indicates that the tumor cells have co-opted the hematopoietic homing axis. Thus far, the CXCL12–CXCR4 axis has been implicated in almost every malignancy studied, including many solid cancers and tumors of hematopoietic origin [10, 133]. However, studies with immune-depleted mice bearing human tumor cells [55] have raised questions concerning the role of the CXCL12/CXCR4 in stimulating skeletal metastasis. This may be due to the limited ability of human tumor cells to adapt to growth in a mouse milieu.

The preceding discussion of CXCL12/CXCR4 interactions in breast cancer suggests that this axis may determine why breast cancer cells metastasize to bone. Clearly this axis is involved in the tumor cell homing and metastasis of many cancers. Although many tumors express CXCR4, not all metastasize to sites that are enriched by CXCL12 [114]. The CXCL12/CXCR4 axis may therefore be an important regulator of the metastatic process, but it cannot be the sole mediator [53, 83].

9.9 Interleukin-8

Chemokine biology is expanding rapidly, with over 50 chemokines that mediate a variety of effects through one or more of the 16 different chemokine receptors so far identified [22]. Interleukin-8 (IL-8; CXCL8) is the prototypical member of the CXC-chemokine superfamily and was originally classified as a neutrophil chemoattractant with inflammatory activity [130].

Many cells synthesize and release IL-8 (and other cytokines) in response to injury, inflammation, and other pathological conditions [115], with IL-8 production induced by pro-inflammatory cytokines such as IL-1 and TNF-α [102]. IL-8 also promotes tumor growth, angiogenesis, bone resorption, and metastasis [4, 11].

Two IL-8 receptors, CXCR1 and CXCR2, share 77% amino acid identity and belong to the super family of seven-transmembrane, G-protein-coupled receptors [115]. Ligand-dependent signaling is mediated by heterotrimeric G proteins, resulting in the exchange of GDP for GTP on the α subunit of the Gq protein [125]. CXCR1 and CXCR2 are expressed on the surface of many normal cells such as neutrophils, basophils, lymphocytes, monocytes, keratinocytes, and endocytes [14, 132].

IL-8 is a potent angiogenic factor in non-squamous cell carcinoma of the lung [113] and induces metastasis in myeloma, breast cancer and colorectal cancer [12, 110, 118]. Elevated serum IL-8 levels are correlated with angiogenesis, tumor progression, and poor survival in NSCLC patients [24, 131]. Similarly, elevated serum IL-8 in breast cancer is associated with an accelerated clinical course, a higher tumor load, and liver or lymph node involvement [15].

Animal experiments utilizing human prostate cancer cells with a high propensity for metastasis to bone have confirmed that IL-8 secretion is positively related to induction of angiogenesis, tumorigenicity, and the development of metastases [57]. IL-8 has also been shown to have potent mitogenic activity for cancers such as melanoma, colon cancer, and pancreatic cancer [71, 111, 132] and to be a potent, direct regulator of osteoclastic differentiation and bone resorption [13, 75], independent of RANKL-activation [13]. The effects of IL-8 on osteoclastogenesis are mediated by CXCR1, the only IL-8 receptor that is expressed on the surface of osteoclasts or their precursors [14].

In multiple myeloma, IL-8 induces proliferation and chemotaxis [98]. Because IL-8 is expressed by myeloma cells, bone marrow stromal cells, and endothelial cells [98, 108], IL-8 in multiple myeloma acts by increasing angiogenesis, and/or bone resorption.

In addition, Arenberg and colleagues [6] demonstrated that when SCID mice that bear non-small lung cancer cells are treated with a neutralizing anti-IL-8 monoclonal antibody,

tumor growth was suppressed by more than 40%. This, in turn, was associated with an overall decrease in lung metastases [6]. Interestingly, anti-IL-8 antibody therapy did not inhibit non-small cell tumor growth in vitro [6]; this suggests that the effect of IL-8 in vivo is not autocrine but paracrine, with IL-8 acting in the tumor microenvironment on cells that secrete factors that support tumor development.

Interestingly, cell–cell contact between tumor cells and platelets during experimental bone metastasis causes lysophosphatidic acid to stimulate IL-8 expression, inducing bone metastasis, skeletal tumor growth, and osteolysis (see also Chapter 5) [20]. It is thus apparent that IL-8 stimulates cytokine production in many different cell types, enhancing tumor growth and cytokine-mediated bone destruction. Therefore, metabolic pathways that cause elevated IL-8 levels may also elicit systemic changes in bone resorption and/or tumor progression [15, 56].

9.10 Interleukin-10

Not all inflammatory interleukins induce tumor progression and bone destruction. For example, the effects of IL-10, a multifunctional cytokine with both immunosuppressive and anti-angiogenic functions [99], oppose those of IL-6 and IL-8. IL-10 through rather ill-defined mechanisms [106], inhibit NF-κB activation and the production of pro-inflammatory cytokines, e.g., TNF-α, IL-6, and IL-12 [72, 99]. IL-10 thus appears to inhibit tumor development and progression. Overexpression of IL-10 in the tumor microenvironment may catalyze cancer immune rejection and suppress tumor development [84].

Erdman et al. [33] have proposed that IL-10 dependent anti-tumor activity is linked to the $CD4^+CD25^+$ regulatory T cells (Tregs). Naturally occurring Tregs inhibit immune pathologies and autoimmune diseases in vivo, possibly because they regulate T-cell number and manipulate the cytokine milieu [94], mechanisms likely involved in tumor suppression as well. These have been identified in mammary and ovarian carcinoma xenografts, where IL-10 expression has been shown to inhibit tumor growth and metastasis significantly [60, 64, 74]. IL-10 also modulates apoptosis and suppresses tumor angiogenesis [60, 64], perhaps via anti-angiogenic changes in the tumor stroma [18]. The potent inhibition of VEGF, TNF-α, and IL-6 production by IL-10 (secreted from tumor associated macrophages) likely contributes to the well-documented inhibitory effect of IL-10 on tumor angiogenesis [46, 62].

As with data concerning most cytokines, not all findings regarding IL-10 action agree. For example, IL-10 has been reported to stimulate tumor growth [39, 72]. Moreover the ability of IL-10 to suppress adaptive immune responses may be a mechanism by which IL-10 supports the escape from immune surveillance [84].

IL-10 expression has been implicated in causing the immune deficiency of cancer patients. In support of this concept, it has been demonstrated that high IL-10 levels in ascites fluid and serum from ovarian cancer patients were positively correlated with histological grade of the tumor, suggesting that IL-10 levels could be a prognostic factor in ovarian cancer [90]. Other studies, however, do not support the inference that selected IL-10 gene polymorphisms are associated with the risk for ovarian cancer [23]. Indeed, low IL-10 expression genotypes have been associated with poor outcome in melanoma patients [81].

The confusing scenario of opposing activities of IL-10 may be due to the fact that IL-10 (or any other cytokine) does not function in isolation, but that its activity is the result of a series of integrated cellular interactions that can lead to either tumor suppression or progression.

9.11 Interleukin-12 and -23

IL-12 and IL-23 are members of a small family of pro-inflammatory heterodimeric cytokines [66]. Both cytokines share a common p40 subunit that is covalently linked either to a p35 subunit to form IL-12 or to a p19 subunit to form IL-23 [66]. The IL-12 receptor is comprised of an IL-12Rb1 and IL-12Rb2 subunit, and the IL-23 receptor of the IL-12Rb1 subunit and a novel subunit IL-23R10 [66]. Both cytokines are

co-expressed by activated dendritic and phagocytic cells, whereas the receptors for both are found on the surface of T cells, NK cells, and NK T cells [72]. Low concentrations of IL-23 receptor complexes are found in monocytes, macrophages, and dendrites [96]. Notwithstanding their similarities, IL-12 and IL-23 are involved in divergent immunological pathways, summarized below.

9.11.1 Interleukin-12

IL-12 is a multifunctional cytokine, the properties of which bridge innate and adaptive immunity. It is a key regulator of cell-mediated immune responses because it induces T helper 1 cell differentiation [29]. IL-12 induces IFN-γ production and proliferation, and the cytolytic action of both NK and T cells. As such, IL-12 is a potent stimulator of innate cellular immunity [29].

Endogenous IL-12 provides resistance to tumors [72], with the anti-tumor activity of IL-12 well-documented in numerous murine models of cancer, including melanomas, mammary carcinomas, colon carcinoma, renal carcinoma, and sarcomas (see [25] for a review). IL-12 inhibits tumorigenesis and induces regression of established tumors, primarily by inhibiting angiogenesis. IL-12 also has potent in vivo anti-tumor and anti-metastatic activity against murine tumors [29]. Interestingly, the efficacy of IL-12 was greatly reduced, but not completely abolished, in immune-incompetent mice [93]. Subsequently, Voest et al. [122] discovered that the anti-angiogenic property of IL-12 depended on its induction of interferon (IFN)-γ expression and that administration of IFN-γ reproduced the anti-angiogenic effects of IL-12. Moreover, IL-12 treatment suppressed osteoclastogenesis [105] and reduced the production of metalloproteases. The latter play a role in mediating remodeling of the extracellular matrix and are required for neo-angiogenesis. IL-12 production is inhibited by IL-10 and TGF-β1, which suppress transcription of the IL-12 p40 subunit and thereby limit the amount of biologically active p70 heterodimer [29].

Thus the anti-tumor effects of IL-12 appear to be mediated by IFN-γ, which, in turn, modulates the anti-angiogenic effects. Interestingly, the increase in TGF-β (due to increased bone resorption of osteolytic metastases) may diminish endogenous IL-12 anti-tumor activity. Conversely, the IL-12-induced IFN-γ production may act on osteoclast-mediated bone resorption via changes in TNF-α and RANKL [38].

9.11.2 Interleukin-23

IL-23 appears to promote the pro-inflammatory function of a memory T-cell subset termed Th17. These Th17 cells are characterized by the production of the cytokine IL-17 and develop due to the influence of TGF-β and IL-6, two cytokines known to be present at high levels in the tumor environment [66]. Although its role in Th17 cell development is still under investigation, IL-23 seems to be crucial for the function, survival, and propagation of Th17 cells [66].

Similar to the IL-12/IFN-γ pathway discussed above, the pleiotropic effects of IL-23 appear to be mediated almost entirely by IL-17 [65]. IL-17 engages its receptor, IL-17 receptor A (IL-17RA), a transmembrane receptor ubiquitously expressed on the surface of monocytes, stromal, epithelial, and endothelial cells [124]. Ligand binding results in the release of osteolytic/inflammatory factors (e.g., IL-1, IL-6, IL-8, TNF-α, prostaglandin E2), thereby enhancing both the inflammatory and osteoclastogenic cascades. Interestingly, the members of the IL-17R superfamily share remarkably little homology with other classes of cytokine receptors [61]. That IL-23, released by tumors, induces IL-17 is an attractive concept, because IL-17 promotes angiogenesis and induces MMPs, two events well known to potentiate tumor growth [66]. These findings support the correlation of increased IL-17 expression observed in ovarian, cervical, and colorectal cancers [95].

IL-23 has been identified as a cancer-associated cytokine [66]. IL-23, but not IL-12, was significantly elevated in human carcinoma samples compared to the concentration found in normal adjacent tissue from the same individual [66]. Because IL-23 was not elevated in the normal adjacent tissues, the upregulation of IL-23 is likely to be specific to the tumor and

not the result of a predisposing genetic condition [66]. The role of IL-23 in tumorigenesis is underscored by the report that mice lacking IL-23 are resistant to endogenous tumor formation [66]. Moreover, the growth of transplanted tumors is restricted in hosts deficient in IL-23 or IL-23R [66]. Even though IL-23-mediated inflammatory processes may provide a tumor-promoting microenvironment, several studies have demonstrated tumor-inhibitory effects of IL-23 [73, 119].

It seems evident that the tumor microenvironment involves the integration of the many signals that are elaborated in the bone microenvironment by immune cells and by tumor and bone cells (Fig. 9.2). How these signals are integrated and coordinated, and how the tumor cells orchestrate their specific responses is not known. However, the available findings suggest the Th17/IL-23–IL-17 axis is an important therapeutic target to prevent bone destruction associated with T-cell activation in cancer and inflammatory diseases [105].

9.12 Summary

Significant strides have been made in understanding major events in tumor progression since 1971 when then President Nixon declared the "War on Cancer." Advances in cancer detection, prevention, and treatment have led to the identification of the roles specific cells play within the tumor microenvironment.

As discussed in this chapter, activation of immune and inflammatory responses produces cytokines that either stimulate or inhibit tumor growth and progression. By and large, most pro-inflammatory cytokines promote tumor development, although this is not the only response. The identification of autocrine and paracrine effects of factors produced within the local tumor microenvironment adds to the complexity of the milieu. To advance the field a more integrated approach is required. Rather than continuing to develop models that segregate the tumor microenvironment from inflammatory cytokines in bone, tumor biologists should study the complexity of factors that orchestrate cell–cell interactions and osteolysis in bone. The integrated response of cells (tumor, immune, and bone) within the milieu of secreted (and matrix) components in the bone marrow microenvironment represents an elegant system in which individual cells respond in a coordinated fashion to enhance tumor cell development and/or survival. These complex processes, and the potential to influence them beneficially in a clinical setting may provide important insights for developing novel therapeutics and represent areas of research that warrant further investigation.

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10.

Prostate Cancer Bone Colonization: Osteomimicry in the Bone Niche

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Abbreviations

PCa	prostate cancer
PSA	prostate-specific antigen
OC	osteocalcin
BSP	bone sialoprotein
RANKL	receptor activator of NFκB ligand
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
HPSE	heparanase
ROS	reactive oxygen species
β2-M	β2-microglobulin
PKA	protein kinase A
EMT	epithelial-to-mesenchymal transition
NED	neuroendocrine differentiation
GFs	growth factors
ECMs	extracellular matrices
CREB	cyclic AMP-responsive element binding protein
FGF	fibroblast growth factor
VEGF	vascular endothelial growth factor
TGF-β	transforming growth factor β
BMPs	bone morphogenetic proteins
PIN	prostate intraepithelial neoplasia
FAK	focal adhesion kinase
MMPs	matrix metalloproteinases

10.1 Introduction and Chapter Overview

Maintenance of a healthy skeleton requires a stable relationship between osteoblast and osteoclast activity. When cancer cells spread to bone, they interact with the bone cells to express bone-like proteins and participate with bone cells in inducing osteoclasts and osteoblasts, thereby bringing about a metabolic imbalance between osteoblasts and osteoclasts. This imbalance may favor osteosclerotic/osteoblastic processes, as in prostate cancer (PCa) or osteosarcoma, or activate osteolytic processes, as in breast cancer and myeloma. The ability of cancer cells to undergo phenotypic changes that allow them to form bone is termed "osteomimicry" and permits cancer cells to home, adhere, and migrate to bone, to invade bone space, and then to utilize the rich bone microenvironment to proliferate and survive because of the abundance of growth factors (GFs) and extracellular matrix molecules (ECMs) found in the bone niche (see Chapter 1). By enhancing angiogenesis in the local tumor microenvironment, these molecules not only help cancer cells to multiply in bone, but also to metastasize to other soft tissues. In short, cancer

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cell osteomimicry amplifies cancer growth at the expense of the bone cells sequestered in the niche.

This chapter aims to (1) develop the concept of osteomimicry and to describe some of the unique signaling pathways involved in osteomimicry; (2) describe cancer cell plasticity and to analyze its evolutionary significance; (3) stress the role of the "cancer cell-derived" receptor activator, NFκB ligand (RANKL), in increasing bone turnover, thereby facilitating cancer growth and survival in bone and ultimately, by stimulating angiogenesis, causing cancer cells to spread to soft tissues; and (4) discuss how osteomimicry supports signal amplification that leads to PCa disease progression. Much of this chapter will focus on PCa cells, because these cells are the true "masters" of osteomimicry. Unlike many other cancers which form osteolytic or mixed bone lesions, PCa cells can literally fill bone marrow with solid mineralizing tumor in late-stage disease.

10.2 Phenotypic Alterations in Prostate Cancer Cells Growing in Bone: A New Signaling Triad

In cancers, metastases often appear long after the first diagnosis of the disease (see Chapter 1). Yet with current techniques, small numbers of PCa cells can be detected in bone [35, 49] even when the serum prostate-specific antigen (PSA) level is very low (<0.1 ng/ml). Conceivably, dormant disseminated cancer cells, when in contact with bone factors or cells in the niche, are activated, proliferate, and become dominant [35, 49]. A key to controlling the spread of PCa cells in bone would be to identify the steps in cancer cell activation. To determine how transdifferentiation of PCa cells occurs in bone, the interactions of PCa cells and bone cells have been studied in animal models that recapitulate human PCa progression [12, 14]. Such studies require the use of cancer cell lines that possess phenotypic characteristics at each stage of the disease: early-stage mimics that are androgen-regulated and more epithelial, and late-stage cells that are androgen-independent and more mesenchymal in nature.

Cell lines available for study include LNCaP, a cell line isolated from human PCa lymph node metastasis, and an ARCaP (*a*ndrogen-*r*epressed *ca*ncer of the *p*rostate) model isolated from the ascites fluid of a male who harbored PCa bone and soft tissue metastases [53, 57, 61]. LNCaP cells fail to form tumors in mice unless co-inoculated with bone-like extracellular matrices or stromal fibroblasts [53]. The in vivo growth of LNCaP tumors in the presence of these inductive molecules results in the "evolution" or "selection" of highly tumorigenic and metastatic LNCaP sublines, C4, C4-2, and C4-2B. These cells, in turn, when implanted in mice, give rise to effective models of human PCa [53, 54]. ARCaP cells are tumorigenic, but a subline, $ARCaP_E$, with an epithelial phenotype, is marginally metastatic when administered intracardiacally. On the other hand, when ArCaP are in contact with cells in the bone niche, they undergo a morphologic and biochemical transition toward a mesenchymal phenotype, $ARCaP_M$. $ARCaP_M$ cells have dramatic bone metastatic potential and produce bone metastases in mice 100% of the time [57]. With the aid of a broad range of analytic approaches, it has been possible to establish numerous links between the osteomimetic properties of human PCa cells and factors and motifs in the PCa bone niche, a linkage likely to mediate the cell signaling cascades that regulate and confer osteomimicry.

One key and unanticipated finding is that PCa, prostate stroma, endothelial, and inflammatory cells secrete a soluble factor, β2-microglobulin (β2-M), which activates downstream signaling of cAMP-dependent protein kinase A (PKA) via the cAMP-responsive element binding protein (CREB). As a result of this activation, PCa cells synthesize and deposit bone-like proteins, including osteocalcin (OC) and bone sialoprotein (BSP) [28, 29], both hallmarks of osteomimicry. β2-M seems to be a major GF and signaling molecule that promotes five aspects of cancer growth and progression in the bone environment: (1) PCa cell proliferation via an increase in cell cycle regulators such as cyclins A and D [28]; (2) PCa cell

survival, via enhanced VEGF-survivin, signaling and androgen receptor (AR) expression [27, 56]; (3) PCa cell adhesion to bone matrix proteins via upregulation of key integrin molecules [18, 19]; (4) PCa cell interaction and colonization of bone via increased RANKL expression [60]; and (5) PCa cell transition from epithelial to mesenchymal (or EMT), a transition that enhances ability to migrate, invade, and metastasize ([43]; see Fig. 10.1).

When PCa progresses from androgen-responsive to androgen-refractory status during disease progression, many other phenotypic features of the cells also change. For instance, production of reactive oxygen species (ROS) in the tumor microenvironment increases [1, 3, 36, 51]. The increase in ROS leads to an increase in overall cell stress and stress responses, which in turn increases synthesis and deposition of extracellular factors, such as matrix proteins, that can further promote disease progression by generating a positive feedback loop. One of these factors produced by PCa cells is β2-M, and a second is the matrix heparan sulfate proteoglycan (HSPG), perlecan. Perlecan is an abundant HSPG in bone marrow and vascular cells (see below), and thus is a desirable therapeutic target to reduce PCa-tumor growth and angiogenesis [11, 17, 21, 47].

Because single marker measures of PCa cell growth in bone are subject to error, there is interest in developing "multiplex" assays for detecting the presence of cancer cells actively growing in bone. Such marker clusters in the long term may prove more useful than single gene products such as PSA which is currently used for PCa screening. In the case of pancreatic cancer, a collection of markers was identified using multidimensional protein identification technology that included CSPG2/versican, Mac25/angiomodulin, IGFBP-1, HSPG2/perlecan, syndecan 4, FAM3C, APLP2, cyclophilin B, β2-M, and ICA69 [41].

One such candidate "molecular trio" for PCa is composed of β2-M, ROS, and perlecan whose interrelationship is depicted in Fig. 10.2. Confirmatory evidence of a triad relationship between β2-M, ROS, and perlecan in clinical human PCa

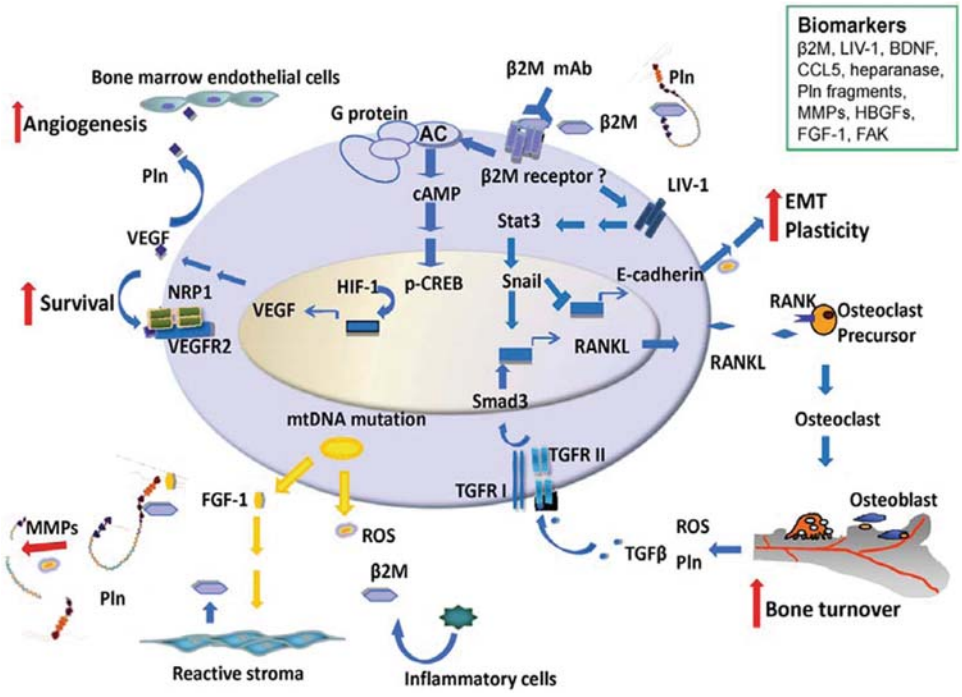

Figure 10.1. The vicious cycles of β2 M-VEGF-survivin, β2 M-pCREB-AR, and β2 M-Snail-RANKL-bone turnover and their contributions to osteomimicry and progression of prostate cancer.

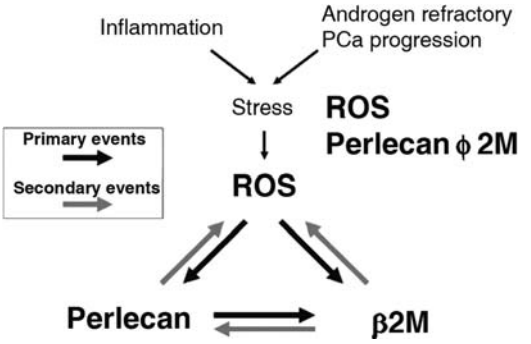

Figure 10.2. Triad relationship among the factors β2 M, ROS, and perlecan including the cascade of events that support cancer growth in the bone.

tissues was recently obtained in our laboratories [1, 2]. To determine if this molecular triad could be used as a measure of PCa disease progression, a series of tissue microarray studies of normal, prostate intraepithelial neoplasis (PIN), benign, primary PCa and PCa bone metastasis were assessed for these markers using immunohistochemistry (IHC) techniques [3, 27, 46, 47]. Other markers of EMT also were included in the study: LIV-1, a zinc transporter associated with EMT and a target gene of β2-M, focal adhesion kinase (FAK), a key cell adhesion molecule and a target gene for ROS and heparanase (HPSE), a heparan sulfate (HS)-degrading enzyme that modulates perlecan activation and angiogenesis in cancer tissues. PCa cells with mitochondrial DNA mutations, associated with increased risk of PCa [9], or activated by β2-M, provoked consistently higher levels of ROS production. ROS stimulated perlecan expression and augmented PCa cell growth in the bone microenvironment. Downstream targets of ROS and β2-M signaling including FAK, perlecan itself, and LIV-1 showed a statistically highly significant relationship to PCa progression from normal, benign, PIN, and PCa-to-PCa bone metastasis (e.g., β2-M ($p < 0.001$), LIV-1 ($p < 0.001$), and FAK ($p < 0.01$)]; HPSE, a HS-dependent modulator of cell signaling, changed distribution in PCa progression consistent with enzyme activation and heparin binding GF delivery ($p < 0.001$). Using logistic regression model analysis, only FAK ($p < 0.022$) and LIV-1 ($p < 0.001$) predicted a Gleason score of 3+3 and 3+4 [1, 2]. Interestingly, recent studies showed that perlecan interactions with PCa cells can activate FAK [20].

Based on the relationships depicted in Fig. 10.2, an integrated model of human PCa progression and EMT has been developed (Fig. 10.3). When normal cells become cancer cells via genetic or epigenetic mechanisms, ROS is produced primarily by stress and secondarily in response to epigenetic regulation by β2-M and perlecan, which together act on heparin-binding GFs. Stromal cells surrounding the cancer cells respond to these inductive "cues", and, undergoing profound structured and molecular transition, become "reactive stroma" [4, 13, 55]. The GF, ECM, and chemokine profiles of "reactive stroma" differ from those of normal "quiescent" stromal cells. The matrix metalloproteinases (MMPs) and HSPG-modulating enzymes, e.g., HPSE, also undergo changes. The interactions between PCa and the reactive stromal cells produce a motile phenotype that metastasizes to lymph nodes and, passing through the vasculature and perineural tissue, reaches bone. Activated PCa cells then use the same routes to establish tertiary metastases at other bone or soft tissue sites. Dissemination is the result of aberrant intercellular communication between tumor and stroma, with β2-M, perlecan, heparin-binding GFs, and RANKL playing crucial roles in affecting the "vicious cycle" between tumor and stroma. This can be made worse by further co-evolution of tumor and stromal cells via genetic or epigenetic changes that lead to increases in angiogenesis, mesenchymal transitions, and increased expression of stress-responsive genes [14, 60, 52]. These changes, in turn, fuel further bone cancer growth, thereby increasing the propensity of bone tumor cells to spread to soft tissues, such as adrenal gland, lung, liver, and kidney [26, 57, 61].

10.3 The Plasticity of PCa Cells. Potential Evolutionary Significance

Transdifferentiation of PCa cells in the tumor microenvironment is similar to what occurs in embryonic development where osteomimicry is

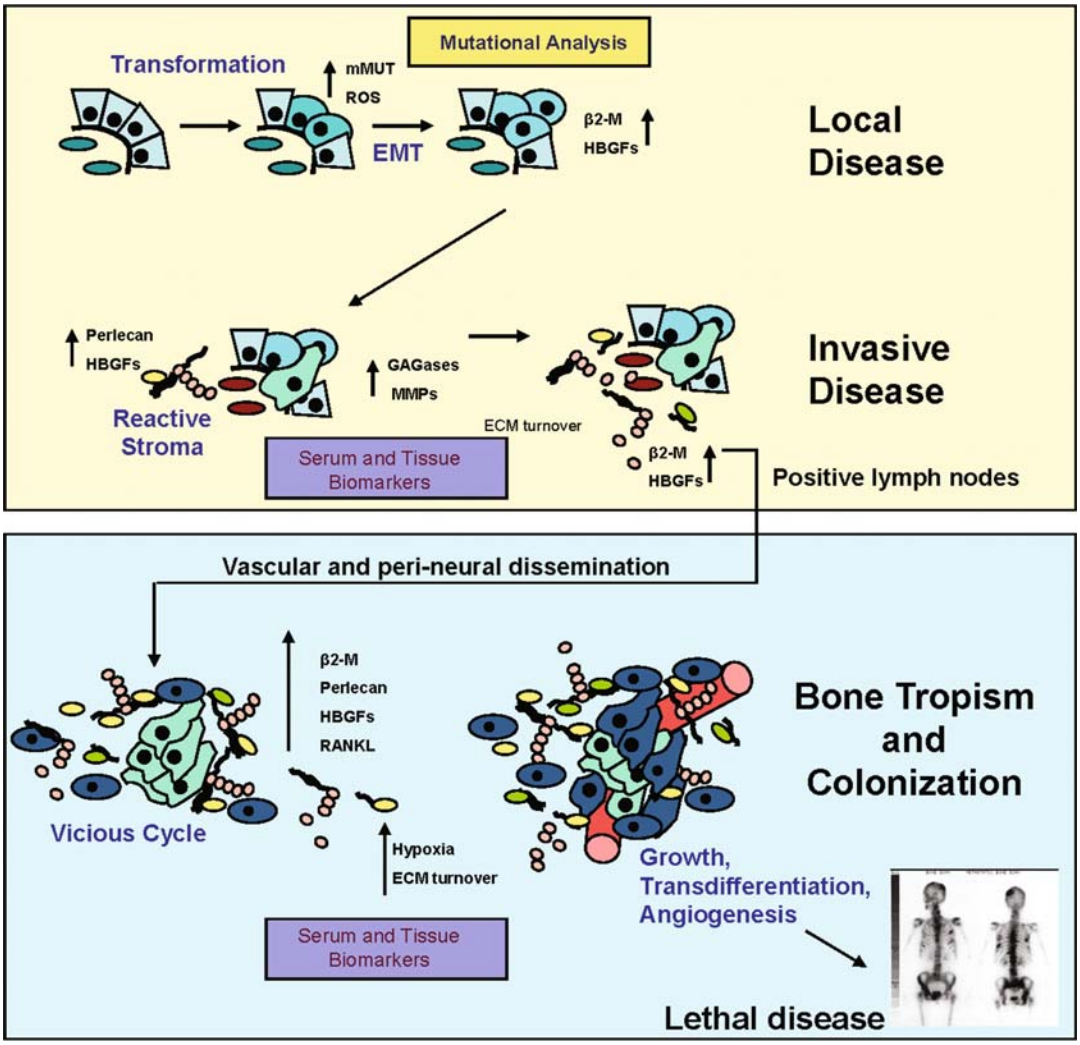

Figure 10.3. Progressive changes leading to EMT and lethal prostatic cancer disease.

regulated by sonic hedgehog signaling [6, 50]. Moreover, transition from epithelial to mesenchymal phenotype often is associated with increased cell migration and invasion [57, 60]. Osteomimicry or EMT are unlikely to occur in normal adult organs, but can be activated in cells undergoing malignant transformation. Nevertheless, not all cells respond to the β2-M signal with osteomimicry (as in the LNCaP model) or with EMT (as in the ARCaP model). Rather introduction of the hypoxia-inducing factor 1α (HIF-1α) in a LNCaP culture will induce EMT [31, 38]. Likewise, osteomimicry can be induced in ARCaP cells when they are subjected to epigenetic factors that regulate key transcription factors (Fig. 10.1). Cancer cells also can adopt other host tissue properties. For example, hypoxic and ischemic cancer cells can induce a vascular endothelial network [24, 25]. When cancer cells transition to mesenchymal cells during development, their epithelial morphology is lost, either partly or completely. They also no longer express epithelial markers such as cytokeratins and Epi-CAM, while expressing mesenchymal markers, vimentin, and N-cadherin. When deprived of serum, cancer cell cultures can express a neuroendocrine phenotype which can grow and survive under certain

pathophysiological conditions [32, 39]. The ability of cancer cells to mimic their microenvironment is critical for the continued growth and survival of cancer cells as they undergo phenotypic change. This refers to hypoxia, nutritional deprivation, exposure to radiation, to chemotherapeutic agents, or to an environment rich in ROS, where chemokines, cytokines, and neuroendocrine factors are released into the microenvironment. The ability of human cancer cells to adapt to changing environments bears remarkable resemblance to what happens when bacteria are subjected to glucose or other nutrient deprivation. When that occurs, bacteria undergo profound genomic and phenotypic changes that have been termed "adaptive" mutation [22, 23, 34]. Conceivably cancer cells, like bacteria, survive under normally unfavorable conditions, at the same time expressing osteomimicry, vasculogenic mimicry, neuroendocrine differentiation (NED), EMT, and other adaptive mutations that allow them to survive and thrive. The diversity of these changes and the heterogeneity of the cancer phenotype make it reasonable to take into account the plasticity of PCa cells in connection with diagnosis, prognosis, and treatment.

10.4 The Functional Role of Cancer Derived RANKL in Osteoclastogenesis

Integrity of the mature skeleton depends on the rates of bone formation and bone destruction remaining relatively equal. Cancer cells may secrete a factor or factors that damage this balance in favor of osteoclastogenesis or osteopetrosis. Breast and PCa cells secrete parathyroid hormone-related peptide (PTHrP) in response to TGF-β [37, 42, 45], causing the osteoblasts to express RANKL. The interaction of RANKL–RANK causes bone turnover to increase and TGF-β from bone cells fuels additional production of PTHrP by cancer cells [37, 42, 45]. Some cancer cells actually secrete TGF-β [7, 10, 59] or BMPs [59], which, in the case of PCa cells, may enhance both osteolytic and osteoblastic processes. Moreover, cancer cell-derived RANKL hastens the maturation of hematopoietic pre-osteoclasts, which in turn promote bone turnover [60]. Figure 10.4 depicts the sequence of events that results when osteo-

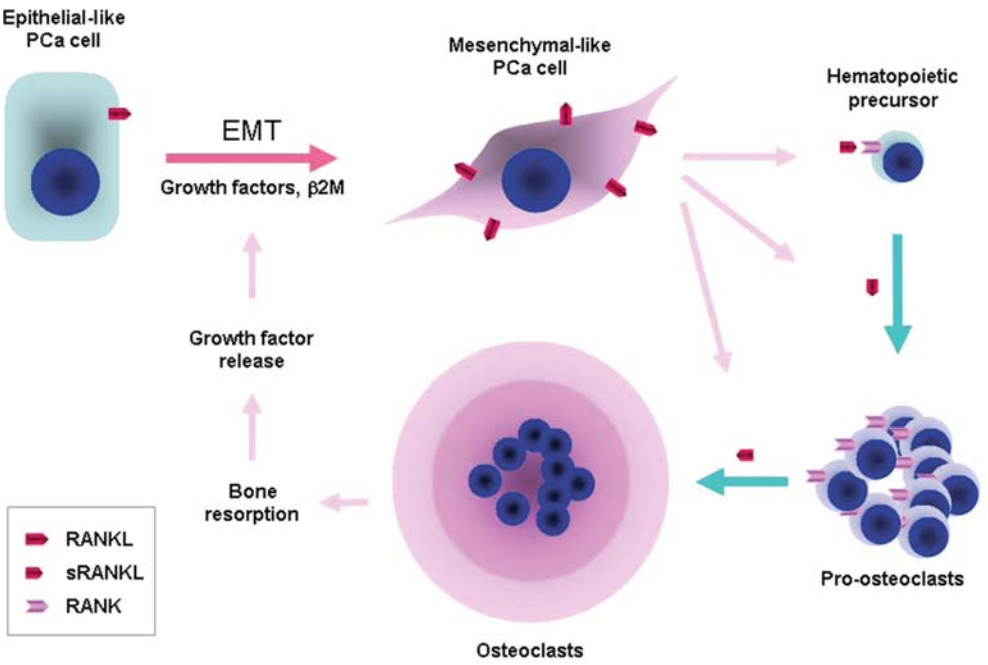

Figure 10.4. Temporal relationship between β2 M and RANKL during prostate cancer bone colonization.

clastogenesis due to PCa cells leads to increased osteoblastogenesis, a prerequisite for new bone formation and PCa growth in the skeleton.

The CREB is one molecule that regulates RANKL in osteoblasts, [5, 58]. This may also be true for PCa cells, but it is equally possible that RANKL in PCa cells utilizes a different mechanism. One reason is that OC expression in PCa cells involves a different mechanism from that in osteoblasts [29].

10.5 Perlecan Supports Signal Amplification Leading to Osteomimicry and PCa Disease Progression

As PCa progresses, PCa cells undergo heterogeneous genotypic and phenotypic changes that make it difficult to treat the cancer with a single modality. Two avenues of transdifferentiation supported by paracrine interactions with bone stroma are osteomimicry/EMT and NED. The latter has been used clinically to evaluate cancer risk [15, 33]. How ECM, including perlecan and HS, supports transdifferentiation is not known; therefore, a therapeutic approach has not been developed. In contrast, the presence of a rapidly growing tumor dramatically increases stromal expression of perlecan in the "reactive stroma" (see above). TGF-β signaling in reactive stroma of PCa is both angiogenic and tumor promoting and appears mediated through a TGFβRII/Smad3-dependent upregulation of FGF-2 expression and release [44]. In bone marrow, perlecan is expressed constitutively at high levels and is the most abundant HSPG in the bone marrow ECM. Strong biochemical evidence has shown that TGF-β affects proteoglycan synthesis both quantitatively and qualitatively and that it can alter HS function by altering the HS-synthesizing machinery [11, 21]. Perlecan is upregulated at the transcriptional level by TGF-β [21, 48] and apparently by increases in ROS (unpublished). The human perlecan promoter contains a number of consensus elements, including those for binding Smad3, CREB, and NFκB, all part of the triad network supporting PCa disease progression.

β2M, at physiological concentrations, releases MMP-1 without release of TIMP-1[41]. This leads to uncontrolled lysis of ECM. BMP1/TLL are produced by alternative splicing of the *TLL1* gene that cleave fibrillar and non-fibrillar collagens, certain GFs, α_2-macroglobulin, lysyl oxidases, laminin, and several proteoglycans, including perlecan [30]. This in turn leads to the release of its derivative, endorepellin [8]. ROS increase production and activate MMPs [16] which degrade perlecan and other matrix molecules present in bone.

Upon disease progression PCa cells acquire plasticity to overcome unfavorable survival conditions. Genomic and epigenomic changes, through coevolution of tumor and stromal cells, support continued growth and survival of cancer cells in bone. The plasticity of PCa cells resembles that of the bacteria under nutritional deprivation. Because bacteria as well as PCa cells undergo genomic and epigenomic adaptation, it may be possible to target "adaptive" changes and plasticity of PCa cells by way of a novel approach to treat PCa progression.

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11.

Bone Pain Associated with Cancer Metastasis

Patrick W. O'Donnell, Nancy M. Luger, and Denis R. Clohisy

In 2005, cancer was second only to heart disease as the leading cause of death in the United States. There are 1.4 million new cases of cancer diagnosed each year resulting in over one-half of a million deaths. Due to improvements in detection and treatment, longer patient survival after diagnosis is now possible. Unfortunately, the quality of life in patients dealing with advanced cancer is significantly reduced. A major contributor to the overall decreased quality of life in such patients is bone cancer pain [12, 45].

Bone cancer can be either a primary cancer or secondary cancer, representing metastatic disease. As metastatic bone tumors are over 200 times more common than primary bone tumors, the vast majority of bone cancers in adults are metastases. Bone cancer pain is the most common source of pain in patients with cancer, and over two-thirds of cancer patients have pain severe enough to require treatment at some point in their lives. Bone cancer pain significantly affects the quality of life of cancer patients [21, 64] (Fig. 11.1). Seventy percent of patients with advanced breast or prostate cancers will develop skeletal metastasis, and the majority of them will experience bone cancer pain [1, 34].

In addition to causing significant pain, bone metastases can cause a host of other devastating problems including fractures, spinal cord compression, bone marrow failure, and hypercalcemia. The rate of fractures in cancer patients with metastatic bone lesions is most directly related to metastatic disease from osteolytic cancers such as myeloma or breast cancer [12]. Fractures secondary to cancer involvement of the bone create very significant problems due to the severe nature of the pain associated with such fractures and the difficult problems associated with fracture repair through weak, tumor-ridden bone. These pathologic fractures not only add to the pain associated with bone cancer but also complicate the overall patient care strategy by causing disability and immobility. A common site of these fractures is the spinal column. Vertebral fractures secondary to bone metastasis are a significant problem owing to the resulting pain and potential neurologic injury. Even without fractures, large bone tumors can lead to severe bone pain, spinal cord compression, and neurologic impingement.

11.1 Bone Cancer Pain

Within the realm of cancer biology, the primary tumor is often not the source of patients' symptoms. Rather, tumor cell metastases to distant organs often prove to cause the majority of cancer pain. Bone cancer pain is frequently

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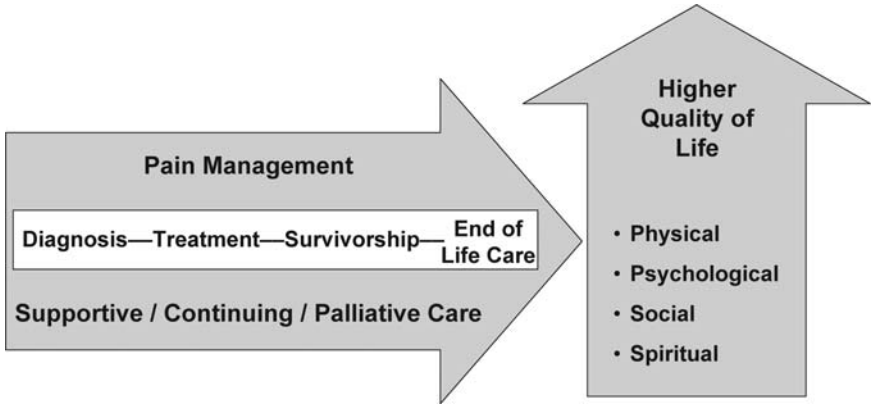

Figure 11.1. Bone cancer pain significantly affects the quality of life of cancer patients. Reprinted with permission from *Cancer Facts and Figures 2007*. Atlanta: American Cancer Society, Inc.

the presenting symptom which indicates cancer metastasis. Cancer-induced bone pain is a complex clinical condition without an easy therapeutic solution.

The development of bone cancer pain typically occurs by one of three mechanisms. First, tumor-induced bone pain is mostly related to direct tumor infiltration into nerves within the bone. The considerable sensory innervation of bone and surrounding tissues is known to contribute to the pathophysiology of bone cancer pain. Due to the rich nervous system innervation, tumors can cause injury to nerves directly and/or by stretching of the periosteum, causing nociceptor stimulation and pain [25, 46, 49, 71]. Second, bone cancer pain may arise due to diagnostic or therapeutic procedures such as biopsy or surgical fixation with orthopaedic implants used to treat or prevent pathologic fractures. Third, bone cancer pain may occur as a consequence of the direct toxicity of cancer therapy. Examples include pain related to chemotherapeutic toxicities or other oncology-related treatments such as administration of growth factors for hematopoietic support [49, 64].

While the natural history varies from patient to patient, bone cancer pain is the most common source of pain in patients diagnosed with cancer. Patients describe bone cancer pain as intermittent at onset, but with progressive development of continuous pain accompanied by breakthrough episodes of very severe pain. Breakthrough pain can be provoked by movement, but it may also be spontaneous and unrelated to movement. Breakthrough pain is often acute, unpredictable, and particularly debilitating. Spontaneous episodes of breakthrough pain are assumed to be related to failure of bone remodeling [10, 50, 71].

Many patients with bone cancer pain experience mechanical allodynia, [34] a particularly devastating pain condition. Mechanical allodynia occurs when a mechanical stimulus not normally perceived as noxious is painful. In patients experiencing this type of pain, even simple touch can be excruciating. This form of pain is often unresponsive to conventional pain therapies [50, 71].

Bone cancer pain becomes increasingly difficult to manage with duration of symptoms and with disease progression. The frequency and intensity of bone cancer pain increases with advancing disease such that 75–90% of patients with advanced-stage disease experience significant daily pain. As one may imagine, patients report that the development and progression of bone cancer pain is associated with a decrease in the quality of life, with immobility, and depression [10, 50, 65, 71].

11.2 Treatment of Bone Cancer Pain

The current treatment of bone cancer pain requires an interdisciplinary approach. Unfortunately, even such an approach can

be inadequate and permanent resolution of bone cancer pain often is not achieved. Treatment currently focuses on eradication of bone tumors, decreasing the impact of tumor-induced bone loss, surgical stabilization of fractures, and pain medications. Chemotherapy and radiation therapy are used to eradicate or reduce the size of bone tumors. Radiation treatment is usually a good means for reducing bone cancer pain. Radiation is successful in relieving pain in 90% of patients, with 50% reporting complete relief of pain. Unfortunately, 50% of these patients experience a relapse in pain symptoms [40, 41, 49].

Other treatments can also be used to eradicate bone cancer pain. Radiofrequency tumor ablation and radiopharmaceuticals (strontium-89 or samarium-153) have shown some promise. Newer tumor-targeted experimental treatments using immunotherapy (cytokine therapy, monoclonal antibodies, and tumor vaccines), hormonal therapy, and small-molecule inhibitors may assist in reducing bone cancer pain [40, 41, 49, 62, 70, 74].

Treatment focusing on decreasing cancer-induced bone loss has shown promise for reducing tumor-induced pain. Successful treatments can be medical and/or surgical. Treatment with bisphosphonates in certain patients has shown up to 50% decrease in patient-reported pain scores [74]. Bisphosphonates have a chemical structure that is similar to pyrophosphate, a normal part of the mineral phase of bone. Bisphosphonates function by inhibiting osteoclast recruitment to the bone surface, reducing osteoclast binding to bone, and inducing apoptosis (programmed cell death) in osteoclasts. In cancer-induced bone pain, the success of bisphosphonates is thought to occur through inhibition of osteoclast-mediated bone loss, specifically in tumor-infiltrated bone.

Surgical stabilization of bones with painful tumors may reduce pain and can prevent pathologic fracture (Fig. 11.2). In the course of management, one must make an assessment of the patient's pathologic fracture risk. The development of a pathologic fracture not only complicates and increases the cancer-related bone pain, but also significantly increases the overall patient mortality and morbidity. Unfortunately, reproducible assessment of the fracture risk secondary to bone cancer is difficult and other medical factors associated with the patient's condition and prognosis must be integrated into any decision regarding surgery [12, 40].

The final and most common means for managing bone cancer pain is through medications. Various combinations of anti-inflammatory drugs, narcotics, and other analgesics can be effective [63]. Bone cancer pain often responds well to opioids, albeit at significantly higher doses than those used outside of oncology. Systemic medical management is particularly poor in addressing breakthrough pain. Limitations such as the side effects of narcotics or other medications when taken at high doses (opioids, NSAIDs, etc.) can have major effects on the adequacy of pain relief. For example, non-steroidal anti-inflammatory medications increase the risk of gastrointestinal bleeding and cardiovascular events, whereas increasing opioids may cause constipation, reduced cognitive acuity, and respiratory depression [33, 40, 50, 62, 63].

New treatment modalities are desperately needed for bone cancer pain. Advances in early cancer detection and cancer treatment have increased the length of survival in many cancers. Even though the length of survival has increased, the patient-reported quality of life during advanced stages of cancer has not improved. New treatments need to be developed and will depend upon development and study of novel animal models that recapitulate bone cancer pain experienced by patients during advanced stages of cancer. With the aid of animal models, the cellular and molecular mechanisms responsible for causing bone cancer pain can be identified. With better molecular/cellular understanding of pain mechanisms, patient-specific and disease-specific treatment strategies can be developed.

11.3 Animal Models Used to Study Bone Cancer

Animal models studying the origin and persistence of bone cancer pain at different anatomic sites (femur, humerus, tibia, calcaneus) and of varied histological origin have been validated and developed in both rats and mice [8, 32,

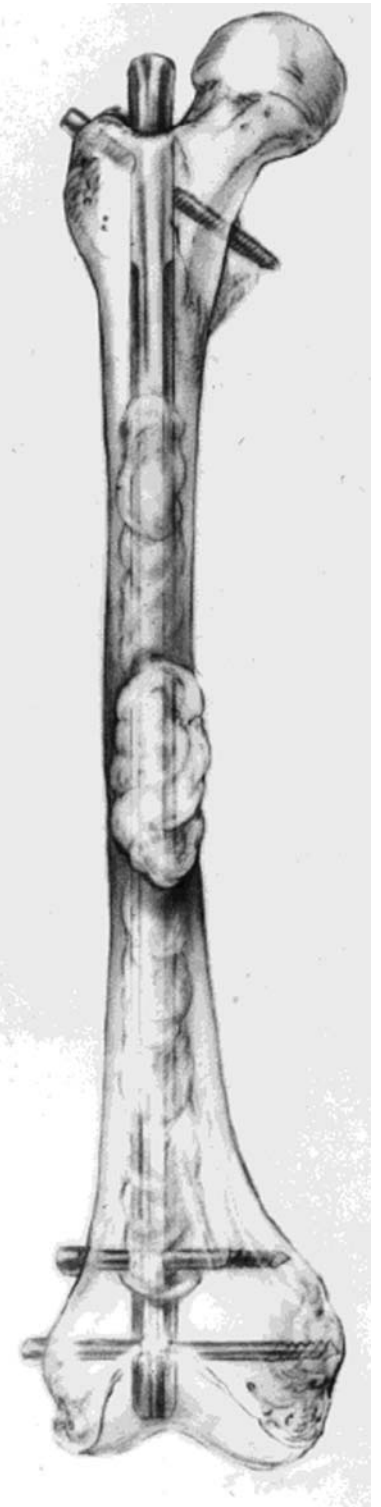

Figure 11.2. Cartoon depiction of a femur with a cancerous lesion located in the diaphyseal portion of the bone. Because the stability of the bone appears compromised by the lesion, an intramedullary nail has been implanted. Reprinted with permission from Harrington [29]. Copyright © 1997 American Cancer Society. This material is reproduced with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

42, 48, 72, 82]. Systemic and local injections of tumor cell lines have been employed to study bone cancer in animal models.

Systemic administration of cancer cell lines has been accomplished via intracardiac or tail vein injections (see Chapter 12). While this model may mimic metastatic spread of cancer cells to bone, the potentially widespread and unpredictable sites of cancer make the model poorly suited to study bone cancer pain. Models using direct injection of cancer cells into the femur, humerus, tibia, or calcaneus have advantages in studying bone cancer pain and have been used widely (Fig. 11.3). The direct injection of tumor cells into specific sites enables reproducible and focused analysis of pain-related behaviors, neurochemical changes within relevant levels of the spinal cord and dorsal root ganglia (Fig. 11.2), bone and tumor histology (Fig. 11.4), and radiographic development of disease. Mice are smaller and thus introduction of tumor cells is more labored and requires a surgical knee stifle. The use of the murine model has been more advantageous secondary to the availability of a larger number of cell lines (e.g., 2472 sarcoma, B16 melanoma, C26 colon adenocarcinoma). Additionally, immunocompromised mice have been used to study human cancer cell lines [16, 53, 58]. Finally, genetically altered knockout mice are in use and offer further advantages for future gene-specific testing and mechanistic study. Rats have a larger bone size which enables introduction of cancer cells via percutaneous injection into the tibia, without violating the knee joint.

Following injection of murine osteolytic tumor cells into the intramedullary canal of the femur, tumor growth and destruction of bone occurs, pain-related behaviors (ongoing and movement-evoked) appear, progressive neurochemical changes occur, and increases in the number and size of osteoclasts at tumor sites is noted. A critical component of this model is to confine the tumor cells within the bone without invading the soft tissues, as this more accurately represents findings in humans. Pain behaviors develop 10 days following injection and continue to escalate until severe impairment occurs between 17 and 21 days. At this point, fractures become evident. Mice are observed for behaviors of guarding the affected limb, decreased limb use of the affected femur, and development of other activity-related pain behaviors. Each of

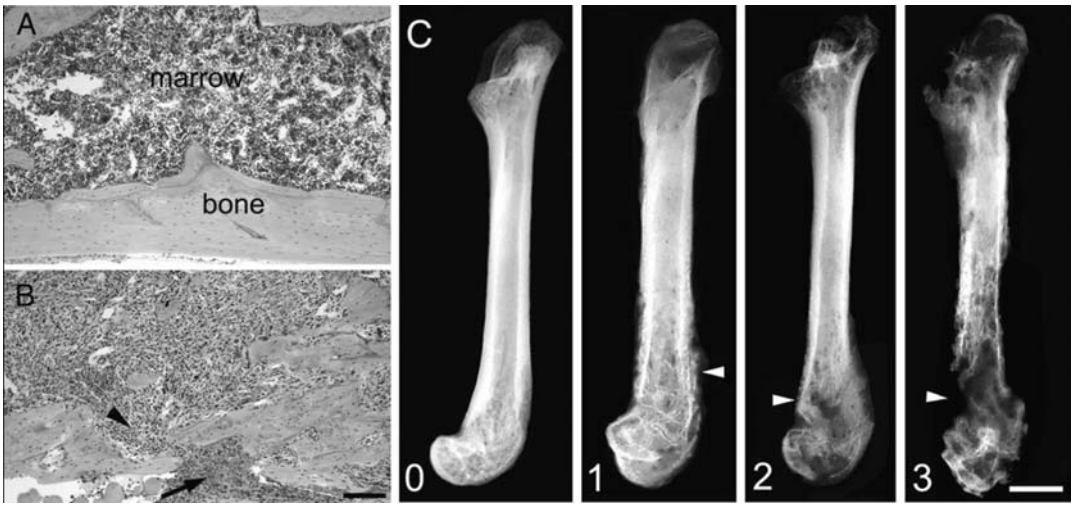

Figure 11.3. Osteolytic bone destruction scoring system after intrafemoral injection of tumor cells. (**A**) Normal bone marrow and intact bone in control femora. (**B**) Tumor-bearing femora illustrating replacement of bone marrow with tumor cells and osteolysis (arrowhead). (**C**) Radiographs highlighting the bone destruction scoring system by showing control bone (0, no loss) and progressive bone loss in tumor-bearing bones (1–3). Reprinted with permission from Schwei et al. [72]. Copyright 1999 by the Society for Neuroscience.

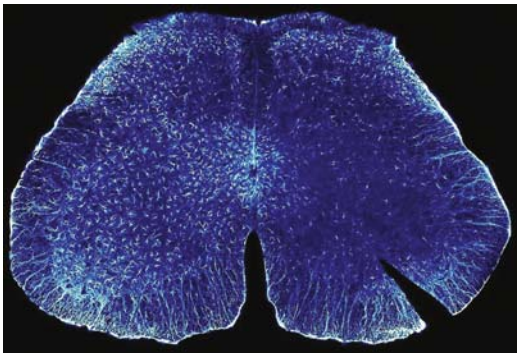

Figure 11.4. Cancer-induced reorganization of the central nervous system. Chronic cancer pain can cause significant alterations in the central nervous system. Confocal imaging of glial fibrillary acidic protein (GFAP) expression in the L4 segment of a tumor-bearing mouse spinal cord shows an increase in the number of astrocytes on the left side of the spinal cord, which receives sensory innervation from the tumor-bearing extremity vs. the right side of the spinal cord which is not transmitting painful stimuli to the brain. Reprinted from Luger et al. [41], Copyright 2005, with permission from Elsevier.

these behavioral measures increases with extent of bone destruction and neurochemical measures of pain [9, 10, 44–46]. In the direct injection model, allodynic pain is observed in mice, once significant bone destruction has occurred. At this point, normally non-noxious palpation of the affected limb results in behaviors indicative of severe pain; this behavior also correlates with extent of bone destruction and neurochemical measures of pain.

In the direct injection model, radiographic analysis of bone destruction can be described as osteolytic in nature with a "moth-eaten" appearance at distal and proximal regions of the femur. This finding is similar to what is seen in humans affected with aggressive, painful bone cancers. Histological assessment has shown tumor presence within the intramedullary space, as well as an increase in the number of activated osteoclasts. Many features of the murine direct injection model of bone cancer pain are important as they validate the clinical similarity between the murine model and clinical findings in humans. This experimental model therefore provides a platform for an integrated systems approach to the study of bone cancer pain with the simultaneous assessment of pain behaviors, neurochemical changes, bone histology, and radiography.

Bone contains a rich network of primary afferent sensory neurons and sympathetic fibers embedded in the periosteum, in mineralized bone, and bone marrow [30, 31, 42] (Fig. 11.4). Primary afferent neurons transmit information from the peripheral tissue back to the spinal cord and brain. The cell bodies of sensory fibers which innervate the skeleton are contained in the dorsal root ganglia [56]. There are two major types of sensory fibers: myelinated A fibers and smaller-diameter unmyelinated C fibers. Large diameter A β fibers mostly conduct non-painful stimuli applied to skin, joints, muscles, and therefore normally do not conduct noxious or painful stimuli [19]. Most small-diameter sensory fibers (unmyelinated C fibers and finely myelinated A delta fibers) conduct noxious or painful stimuli and are termed nociceptors [56]. Nociceptors can detect physical and chemical stimuli [6, 35]. They transmit noxious or painful stimuli and respond to factors or conditions that transmit pain. Stimulators include bradykinin, prostaglandins, endothelins, nerve growth factor, and acid condition channels (ASIC, TRPV-1). Study of the possibility that these factors and conditions affect bone cancer pain has greatly increased our understanding of pain generation and has identified potential targets for therapeutic intervention.

Alterations in the normal skeletal remodeling at sites of bone cancer have led to an increased understanding of the complex cellular interactions which occur at sites of tumor and may contribute to bone cancer pain. Histologic and radiographic analysis of osteolytic (bone destroying) tumors in experimental models have consistently demonstrated an increase in mature osteoclasts. Osteoblastic (bone forming) tumor cell lines (ACE-1) have been shown to cause an increase in both osteoclasts and osteoclastic activity. It is noteworthy that both osteolytic and osteoblastic cancers are characterized by osteoclast proliferation and hypertrophy [11].

Pain generation and maintenance may be derived from alterations in normal skeletal remodeling, tumor-released products, and tumor-induced remodeling of the sensory innervation of bone (Fig. 11.5). Targeted therapeutics

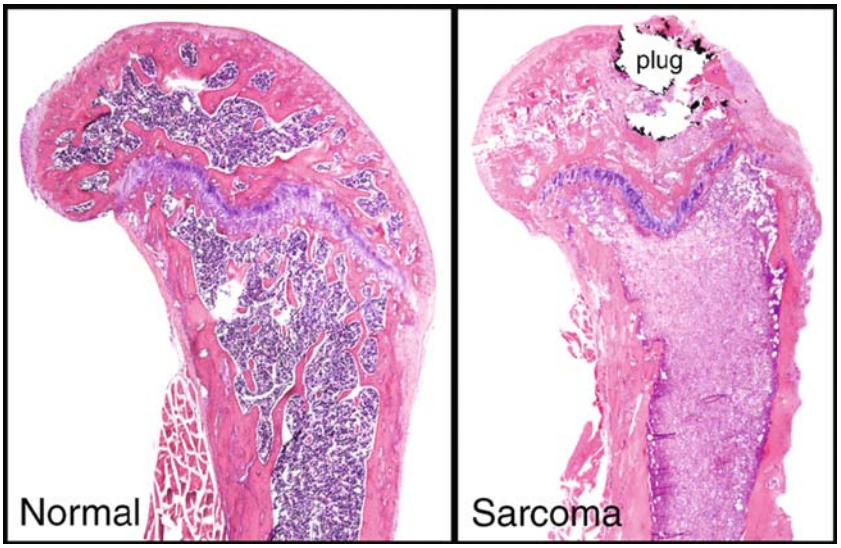

Figure 11.5. Bone destruction following injection of osteolytic sarcoma cells into the intramedullary space of the femur. H&E staining of normal and 17 day sarcoma-bearing femora which illustrates that there is a clear separation of mineralized bone (*pink*) and marrow cells (*deep blue*) in normal bone. In contrast, the intramedullary space of the tumor-bearing femora has been infiltrated with sarcoma cells (*purple*). Reprinted from Luger et al. [41], Copyright 2005, with permission from Elsevier.

using the findings from this work may be used to develop new therapies.

11.4 Therapeutics and Skeletal Remodeling

Studies of the osteoclast-inhibiting agent alendronate in a murine model have shown that bisphosphonates attenuate tumor-induced changes in pain, central and peripheral neurochemical changes, and reduce osteoclast number and activity. Alendronate treatment did not affect tumor burden, as both tumor growth and tumor necrosis were increased [74]. Clinical use of bisphosphonates has been shown to reduce pain in skeletal metastases [7, 23, 43].

Another osteoclast treatment strategy that holds promise for targeting osteolysis and pain is blockade of the RANK–RANK ligand (RANKL) interaction. This has been shown experimentally using osteoprotegerin (OPG), a decoy receptor that binds to RANKL and thus prevents the survival, formation, and proliferation of osteoclasts [14, 52, 79]. Studies in the 2472 sarcoma model have shown a decrease in pain behavior and a significant decrease in osteolysis and pain behavior. The use of this treatment strategy is under way in clinical trials.

The tumor microenvironment is comprised of a variety of cell types, including inflammatory cells, bone cells, hematopoietic cells, and cancer cells. Various pro-inflammatory factors have been shown to be released at sites of painful tumors and are supposed to sensitize or directly excite nociceptors, including prostaglandins, endothelins, interleukins, epidermal growth factor, tumor necrosis factor alpha, and transforming growth factor beta [15, 17, 24, 54, 55, 59, 61, 66, 68, 78, 81, 83–85]. Each of these factors may play a role in the development and maintenance of bone cancer pain.

Cyclooxygenase (COX) isoenzymes are expressed at high levels by both cancer cells and tumor-associated macrophages. The presence of COX isoenzymes produces high levels of prostaglandins [20, 37, 51, 57, 75]. Prostaglandins are synthesized from arachidonic acid by COX-1 and COX-2 isoenzymes and are involved in many processes that occur at sites of bone cancer [28, 39, 47, 67, 76, 80, 86]. These processes include angiogenesis, bone homeostasis, inflammation, and tumorigenesis.

Prostaglandins modulate tumor growth, survival, and angiogenesis. Animal studies using COX-2 inhibitors to treat bone cancer pain have shown a decrease in ongoing and movement-evoked pain, reduced neurochemical changes, and retarded tumor growth [69]. COX-2 inhibitors may provide incremental improvement for humans with bone cancer pain.

Endothelins are expressed by several tumor types (prostate, colon, lung) and are suspected to contribute to the development of bone can-

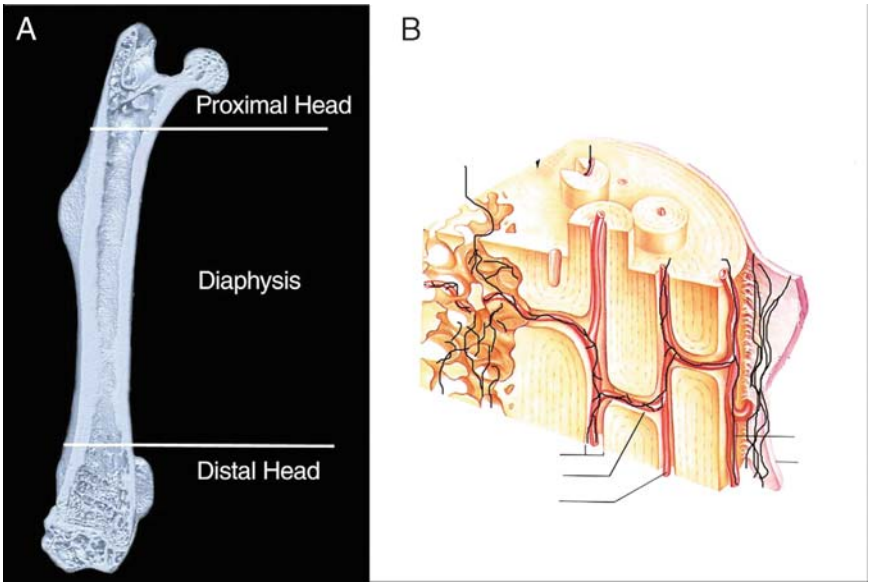

Figure 11.6. Micro-computerized tomography of the mouse femur (**A**). A schematic diagram (**B**) illustrating the sensory fibers within the mineralized bone and periosteum. Note that the periosteum has the densest innervation of sensory fibers. However, taking into account the total volume the sensory and sympathetic innervation, the greatest volume is in the mineralized bone followed by the bone marrow and finally the periosteum. Reprinted from Mach et al. [42], Copyright 2002, with permission from Elsevier.

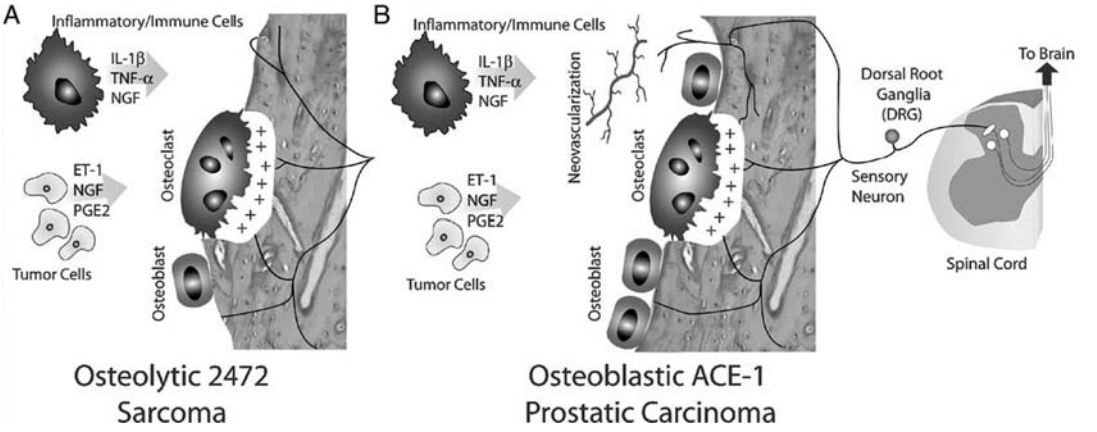

Figure 11.7. Schematic of the sensory innervation of the intramedullary canal and mineralized bone and the bone remodeling that occurs in the sarcoma and prostatic carcinoma (ACE-1) tumors. In both models, tumor or inflammatory cell-released factors may play a role in the generation and maintenance of cancer-induced bone pain and may mediate neurochemical reorganization in the dorsal root ganglia or the spinal cord with advanced disease. Reprinted with permission from Halvorson et al. [26].

cer pain [2, 3, 5, 38]. The biologic activity of endothelin is mediated by two receptor types (ETaR and ETbR). ETaR is expressed on sensory neurons and vascular smooth muscle cells, while ETbR is expressed on nonmyelinating Schwann cells, endothelial cells, smooth muscle cells, and macrophages. Direct application of endothelins onto peripheral nerves has induced pain in animal studies [16]. Administration of the selective endothelin A receptor antagonist significantly reduces ongoing and movement-evoked cancer pain, as well as reduce many of the neurochemical indices of peripheral and central sensitization [60].

Bradykinin is released in response to tissue injury and inflammation and therefore is a potential target for treating bone cancer pain. There are two bradykinin receptors, B1 and B2. B1 receptors are normally expressed at low levels by sensory neurons and are upregulated after

DRUG CLASS	SITE OF ACTION	OSTEOLYTIC		OSTEOBLASTIC	
		PAIN	DISEASE PROGRESSION	PAIN	DISEASE PROGRESSION
TUMOR/INFLAMMATORY PRODUCTS					
SELECTIVE COX-2 INHIBITORS	Prostaglandin synthesis in the CNS and PNS	↓	↓	↔	↔
ENDOTHELIN RECEPTOR ANTAGONSITS	Nerve fibers (CNS, PNS) & smooth muscle cells (PNS)	↓	↔	↓	↔
ANTI-NGF ANTIBODY	trkA receptor in the PNS	↓	↔	↓	↔
ACID SENSITIVE ION CHANNELS (TRPV1; ASIC)	Blockade of H+ ion channels in the CNS and PNS	↓	↔	↔	↔
PURINERGIC RECEPTOR ANTAGONISTS	Blockade of P2X receptors in the CNS and PNS	↓	↔	↔	↔
OPIOIDS	Opiod receptors in the CNS and PNS	↓	?/↑	↓	?/↑
BONE REMODELING					
OSTEOPROTEGERIN (AMG-162)	Inhibitor of osteoclast mediated osteolysis in bone	↓	?/↓	↔	?/↓
BISPHOSPHONATES	Pro-osteoclast apoptotic action in bone	↓	?/↓	↓	?/↓
TRPV1 ANTAGONISTS	TRPV-1 receptor blockade in the CNS and PNS	↓	↔	↔	?
NERVE INJURY					
ANTICONVULSANTS (GABAPENTIN, PREGABALIN)	Regulators of calcium channel activity and GABAergic neuronal discharge in the CNS and PNS	↓	↔	?	?
ANTIDEPRESSANTS (CYMBALTA)	Selective Serotonin and Dopaminergic reuptake inhibitors in the CNS	?	?	?	?
SODIUM CHANNEL BLOCKERS (NAV1.8, 1.9)	Blockade of sodium channels in the CNS and PNS	?	?	?	?
GDNF THERAPY (ARTEMIN)	Stimulation of GDNF receptor in the CNS and PNS	?	?	?	?

Figure 11.8. Mechanism-based therapies currently available or under investigation in animal models of bone cancer pain. Reprinted with permission from Halvorson et al. [26].

peripheral inflammation and/or tissue injury. B2 receptors are normally expressed at high levels on sensory neurons. Animal studies using a B1 antagonist have been shown to reduce ongoing and movement-evoked pain [13, 22, 73].

Nerve growth factor (NGF) has been shown to modulate inflammatory and neuropathic pain states in animal models [4, 36, 77] and may be involved in the survival and proliferation of some tumor cells [18]. NGF is expressed in inflamed and injured tissue. This growth factor activates sensory neurons expressing the NGF receptors trkA receptor or p75. Treatment of mice with anti-NGF sequestering antibodies has provided profound pain relief, but has had no effect on bone remodeling, osteoclast or osteoblast proliferation, tumor growth, or innervation of bone. Shockingly, the reduction in pain observed in mice was as effective or greater than high-dose opioid analgesics [27].

Examination of the mechanisms that drive bone cancer pain suggests that bone cancer pain is associated with damage to sensory neurons. The observed neurochemical changes are similar to those that have been described following peripheral nerve injury. The clinical use of gabapentin and pregabalin in the treatment of chronic neuropathic pain syndromes is increasing and is effective in treating post-herpetic neuralgia and diabetic neuropathy. The exact mechanisms of gabapentin and pregabalin action are not well understood. Animal models of bone cancer pain have shown that gabapentin treatment reduces pain, but has no effect on tumor growth, osteolysis, or neurochemical alterations produced by bone cancer [60]. This suggests that a component of bone cancer pain is due to injury of primary afferent nerve fibers.

The mainstay of treatment for bone cancer pain has been opioid analgesia. However, the doses required for advanced bone cancer pain produce significant side effects. These include sedation, constipation, respiratory depression, and cognitive impairment. Therapies that specifically target the mechanisms responsible for the production and progression of bone cancer pain are needed. Future treatment using a combination of therapies that collectively target multiple mechanisms driving bone cancer pain will likely improve clinical management of this devastating condition (Figs. 11.6, 11.7, and 11.8).

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12.

Small Animal Models for the Study of Cancer in Bone

David J. DeGraff, Fayth L. Miles, Ronald R. Gomes, and Robert A. Sikes

12.1 Introduction

Advanced cancers commonly result in metastasis to bone where interactions between the cancer cell and the bone microenvironment produce predominantly osteoblastic or osteolytic lesions. In humans bone metastasis is observed in 60–85% of advanced cancers from tissues as disparate as prostate, breast, kidney, and lung [9]. In animals, however, spontaneous bone metastases are uncommon and investigation of the patterns of bone metastasis mimicking human cancer dissemination is challenging. Experimental models of bone metastasis consist of injections of cancer cells or tissue orthotopically, intracardially, intravenously (via the tail vein), or directly intraosseously into immunocompromised hosts, mostly mice, and, rarely, other small mammals such as dogs and cats. SCID-hu xenograft models allow for the engraftment of human bone in SCID mice, followed by colonization of cancer cells. This allows direct study of bone colonization with a mostly human bone fragment, intended to reveal histopathological phenotypes that correspond to human bone metastases. Neoplasms that arise spontaneously in mice or rats have been used to establish syngeneic cancer cells: host models where bone metastasis can be studied in the context of a competent immune system. Advances in transgenic technology utilizing newly discovered transforming genes have increased the number of strains that exhibit overall and organ-specific tumor formation. They also have yielded models that simulate some aspects of tumor progression to cancer and distal metastases. Notwithstanding these advances, few of the models exhibit the high incidence of metastases to bone as seen in humans. A variety of human cancer-derived xenografts remain the most widely used to study cancer–bone interaction. Cancer tissue fragments are grown in immunocompromised hosts and established as xenografts. The xenografts, in turn, are analyzed for relevant biomarkers from the tissue of origin. Cell cultures then are established which are passed to the host, either by injection or serial passage of xenografted tumor pieces, in an attempt to produce highly aggressive variants that metastasize spontaneously. Some of these models have shown long latency with a high degree of specificity for metastasis to bone, also leading to spinal compression and paraplegia. An example of long latency is the LNCaP human prostate cancer (PCa) progression model. Other models have short latency, with poor specificity

David J. DeGraff and Fayth L. Miles contributed equally to this work.

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for bone, as is the case for the surgical orthotopic implantation model.

Many growth factors and pro-metastatic factors have a pivotal role in chemotaxis and bone metastasis. Once cancer cells have metastasized to bone, Interactions between bone stromal cells and cancer cells determine the extent of cancer cell growth and bone destruction or formation. Whether a given bone is actively remodeling or quiescent also plays a role in the cancer–bone microenvironment interaction. Few models exist to analyze the cancer–bone interaction in vivo during conditions of active bone remodeling. Our laboratories have used the hindlimb suspension system to examine how alterations in bone turnover affect colonization by tumor cells and whether bone turnover affects transition from cancer dormancy to full-blown metastatic bone lesions. This chapter provides an overview of available animal models that metastasize to bone with high frequency. Where possible, we have included the following categories of induction of bone metastasis: spontaneous, syngeneic, transgenic, and the various routes of human xenograft delivery.

12.2 Breast Cancer

It is estimated that in 2007, 178,480 women were diagnosed with breast cancer (BCa) [66], and that some 40,460 women died from the disease. The median survival of patients with metastatic BCa is 2–3 years. Approximately 80% of patients whose BCa has metastasized develop bone lesions. This makes BCa the number one cancer with metastatic bone involvement [118]. Although 60–80% of BCa samples [79] express the estrogen receptor (ER), ER expression is less prevalent in distal metastases. These are among the reasons why adequate models for the study of BCa metastasis to bone need to be developed.

12.2.1 Models for the Study of Breast Cancer Metastasis to Bone

12.2.1.1 Spontaneous

Spontaneously arising murine mammary carcinomas do not metastasize to bone. However, bone metastasis models have been developed that use spontaneous mammary tumor cells. Some inbred strains of laboratory mice carry Mouse Mammary Tumor Virus (MMTV), or other mutations. These confer benign and malignant neoplasms whose incidence differs in different mouse strains [103]. These models are poorly metastatic and largely ER-negative; as such, they are on the whole poor choices for studying metastatic BCa.

The first model of spontaneous bone metastasis used the 4T1 cell line, a clonal tumor cell line that is derived from a spontaneous tumor, expressing MMTV, in a BALB/cfC3H mouse [27, 53]. This cell line spontaneously metastasizes to the lungs and liver in approximately 1 month [7]. In what is essentially a syngeneic, orthotopic progression model (see next section), injection of these 4T1 cells into the mammary fat pad of BALB/c mice results in metastasis to bone. Two clonal variants of this tumor line, 4T1.2 and 4T1.13, produced overt, spontaneous metastases in the spine and femur. This was profiled using a quantitative reverse transcription polymerase chain reaction (Q-PCR) assay confirmed on tissue sections stained with hematoxylin and eosin (H & E) [82].

The metastatic 4T1.2 and 4T1.13 cell lines are more adhesive to Matrigel, a basement membrane preparation, and much more migratory and invasive than the nonmetastatic sublines. Mice bearing 4T1.2 and 4T1.13 tumors also had higher serum calcium and parathyroid hormone-related protein (PTHrP), a potent stimulator of bone resorption, levels than mice without bone metastases. These findings indicate that bone turnover is significant and that the animals have osteolytic bone disease, as is true for most human BCa [20, 91]. The metastatic cells also had increased expression of a number of genes that encode ECM proteins, including the matrix protein POEM, which, when silenced, reduces metastasis to lung, kidney, and bone [3].

12.2.1.2 Syngeneic

A major benefit of syngeneic model systems is that they allow cancer modeling in an immunocompetent host. They do not, however, allow for an analysis of the determinants of human metastatic disease. A few murine cell lines have

been developed in syngenic models for the study of BCa metastasis.

The Walker 256 carcinosarcoma rat tumor, discovered in 1928, arose spontaneously in the mammary gland of a pregnant albino rat. When Walker tumor cells are injected into the iliac artery of Walker rats, lesions form and the tibia and femur are destroyed after 7 days [67]. Bone lysis can be overcome in these rats by bisphosphonate therapy [67]. When Walker tumor cells are implanted into the femur, bone mineral content decreases, as demonstrated with micro-computed tomography (microCT), X-ray absorptiometry (DXA), and quantitative computed tomography (pQCT). Treatment with the bisphosphonates partially inhibits bone lysis [80, 84, 85, 80, 81]. When bisphosphonate treatment is combined with irradiation, the bone remineralizes and becomes more stable [81]. When Walker 256 Line A cells are injected into the gastrocnemius muscle of rats, the nearby tibias are invaded within 14 days [47].

The 13762 syngeneic rat tumor cell line, implanted in the mammary glands of Fisher 344 rats, metastasizes to lymph nodes and lung [99], and, when implanted in the proximal tibia, these tumor cells induce osteolysis after 7 days, which is suppressed with bisphosphonate treatment. Interestingly, immunohistochemistry demonstrated that these tumor cells express PTHrP and transforming growth factor-beta 1 (TGF-β1), a further sign of their osteolytic nature [5].

12.2.1.3 Xenografts

12.2.1.3.1 Orthotopic Injection

Orthotopic injection [87] of human cancer cell lines is the current method of choice to study cancer metastasis, but bone metastasis in this model is infrequent. Both MDA-MB-231 and MDA-MB-435 cell lines produce tumors when injected subcutaneously, but tumors arise in only about 40% of animals, and the resulting metastasis is very disappointing. However, when these cells are injected orthotopically into the mammary fat pad, 80–100% of nude mice develop metastases within 20 weeks. Common sites of metastatic spread include lymph node and lungs, and to a lesser extent, brain, heart, adrenal glands, and muscle. Distal metastases are established in 70% of mice by 8 weeks [113]. However, MDA-MB-435 cells fail to metastasize to bone after orthotopic implantation.

12.2.1.3.2 Intracardiac Injection

Direct injection of the cloned subline of B16 murine melanoma cells G3.26 into the arterial circulation led to the earliest success in inducing skeletal metastases in mice [6]. Since then, the technique has been modified to inject human cancer cell lines into nude mice. Injection of MDA-MB-231 results in the high frequency and preferential formation of osteolytic bone metastases that can be detected by standard X-ray imaging [69]. By contrast, metastasis to organs other than bone is much less frequent. When MDA-MB-231 cells were transfected with a constitutively active TGF-β type I receptor, PTHrP levels and osteolytic bone metastases were increased. PTHrP appears to have a direct effect on enhancing bone metastasis, inasmuch as injection of MDA-MB-231 transfected with PTHrP cDNA and the dominant-negative TGF-β type II receptor, accelerated bone metastasis and decreased survival [153]. The MDA-MA-MB-231-dervied cell line, SCP2, produced extensive osteolytic lesions in the distal femur, illac crest, sacrum, and vertebral body 8 weeks after intracardial injection into immunodeficent mice. The authors of this study used luciferase-based noninvasive bioluminescence imaging to mointor metastasis, and a bone-metastasis gene expression signature to predict bone-metstaizing BCa cells. Such an approach is clinically useful for prediciting patterns of organ-specfic metastasis in BCa patients [89]. Osteolytic bone metastases of MDA-MB-231 cells after intracardial injection are inhibited by the bisphosphonate, risedronate in nude mice. This bisphosphonate reduces tumor burden selectively in bone, wherease tumor growth in soft tissues is unaffected [120].

12.2.1.3.3 Intravenous Injection

Intravenous (IV) injection of BCa cells into the tail vein of nude mice has led to widespread metastasis and has permitted selecting subpopulations of highly metastatic cells. For example, the bone metastatic BO2 cell line was established

following injection of MDA-MB-231 BCa cells into the tail vein. Because BO2 cells have higher levels of $\alpha_V\beta_3$ integrin, they induce twice as many osteolytic lesions and cause the metastasized bone area to increase fivefold [105].

12.2.1.3.4 Intrafemoral/Intratibial Injection

Intrafemoral/intratibial injection of cancer cells is not appropriate to elucidate early rate-limiting steps of the metastatic cascade, but provide a powerful technique to model the interactions between cancer cells and the bone microenvironment. For example, intrafemoral injection of MDA-MB-435 cells produces tumors that can be detected in less than 4 weeks by standard imaging techniques [138]. Injection into the intratibial artery followed by ligation of the artery delivers the BCa cells directly to the bone microenvironment, and prevents further spread of tumor cells. Osteolytic lesions are visible in 95% of injected animals within 18 days, and tibial bone mineral density is significantly reduced 42 days later [100].

12.2.1.3.5 SCID-hu Mouse Models

The recently developed SCID-hu recombinant models represent a new frontier for the study of in vivo modeling of cancer metastasis to bone. In this model, fetal or adult human bone tissue is injected into SCID mice. This (1) allows modeling the interaction between human cancer cells and bone tissue, (2) provides specificity regarding the preferential colonization of human bone (as opposed to murine bone) by human cancer cell lines, and (3) demonstrates the importance of local factors produced by the bone microenvironment and the metastatic cancer cells.

In one study, Kuperwasser et al. [83] used tissue from discarded femoral heads of patients undergoing total hip replacement. Bone cores were implanted subcutaneously into the dorsal flanks of 5-week-old NOD/SCID mice. The integrity of the implanted human bone tissue was verified.

This should be combined with previous paragraph model was then used to identify BCa cell lines that displayed osteotropism. Only SUM1315 were able to colonize human bone, but there was no metastasis to the lungs, notwithstanding the systemic dispersal of cancer cells by this technique. Interestingly, implantation of SUM1315 into the mammary fat pad resulted in lung and bone metastasis in 12 weeks.

12.2.1.4 Transgenic

There is no transgenic model of BCa that leads to metastatic bone lesions. Regardless of HER-2/neu expression status, the rapid onset and growth of primary tumors, accompanied by extensive lung metastasis, precludes detection of bone lesions [23, 65, 92, 102, 125, 154]. From this it is evident that even cooperative pathways that enhance tumorigenicity and metastasis to the lung do not confer bone metastasis. An alternative approach that facilitates the metastatic spread of mammary tumors to bone is to overexpress PTHrP in the mammary fat pad of mice, in conjunction with treatment with 9,10-dimethyl-1,2-benz-anthracene (DMBA), a known mammary carcinogen [148]. In that study, 84% of K-14 PTHrP transgenic mice had developed mammary tumors within 1 year, as compared with only 59% of control mice. This indicates that PTHrP mice readily developed mammary tumors when treated with DMBA. The transgenic mice tended to develop tumors sooner than controls. They also were hypercalcemic. Twenty-two percent of the transgenic mice displayed overt metastases including lung and liver, but there was no bone metastasis.

12.3 Lung Cancer

Eighty-seven percent of all lung cancers are thought to be tobacco-related [115]. Lung cancer, therefore, is the single most preventable cause of cancer deaths. The World Health Organization estimates that the 5.6 million cigarettes smoked at the close of the twentieth century will cause nearly ten million fatalities per year by 2030 [79]. In the United States some 213,380 new cases of lung cancer were predicted to occur in 2007 [66]. The 5-year survival rate for patients with lung cancer is about 15%, and for those with stage IV disease it is less than 5% [66,79]. Approximately 60% of SCLC and 30–40% NSCLC patients present with stage IV metastatic disease [79], and lung cancer is the fourth most prevalent cancer to establish metastasis to bone

[37,118]. The need for models to study the development, progression, and prevention of metastatic disease is obvious.

12.3.1 Models for the Study of Lung Cancer Metastasis to Bone

12.3.1.1 Spontaneous

Spontaneous lung tumors occur rarely in animals. In mice and rats the rarity of spontaneous lung cancers makes them impractical as metastasis models. In B6C3F1 mice, adenomas occur in about 16% and carcinomas in 5% of the animals [50], but metastases are practically nonexistent [28]. The rates are even lower in rats.

12.3.1.2 Syngeneic

There are very few syngeneic models for the study of lung cancer metastasis to bone. However, subcutaneous injection of a PTHrP-overexpressing cell line referred to as IP, which itself was derived from a spontaneously arising caudal lobe tumor of the left lung in a 25-month-old male F344/DuCrj rat, resulted in severe emaciation, hypercalcemia, hypophosphatemia, a decrease in femur shaft thickness, and an increase in osteoclastic areas [97]. IP cells may therefore actively induce bone resorption, and subclones may successfully colonize bone. In a subsequent publication, the authors derived an IP subclone, termed IP-B12, and injected it into the left ventricle. This led to osteolytic metastases in long bones and vertebrae, followed by osteolytic fractures and nerve compression, and extensive replacement of marrow by tumor cells [96].

Recently, the Lewis lung carcinoma (LLC) model, which consists of cells derived from a spontaneous murine lung cancer, was shown to weakly colonize bone marrow 4 weeks after intravenous injection into syngeneic hosts, when LLC were covered with platelet-derived microvesicles [63]. Conceivably this ability to colonize bone may be amenable to genetic manipulation, so as to increase bone colonization.

12.3.1.3 Xenograft

12.3.1.3.1 Orthotopic

The NCI-H460 cell line was derived from the pleural effusion of a patient with a large cell lung cancer. This cell line has been used in both nude mice and rats [57] and reliably produces skeletal metastasis in up to 75% of animals, 32 days after orthotopic implantation (SOI) of subcutaneously grown tumor pieces into the left lung. To enhance the detection of metastatic lesions in nude mice, AntiCancer, Inc. (San Diego, CA) produced GFP-expressing H460 cells and orthotopically implanted (SOI) subcutaneously grown tumor tissue into the left lung. SOI led to contralateral lung and chest wall metastasis and seven out of eight tumors metastasized to the skull, tibia, femur, and marrow [150]. The metastatic lesions due to these cells were not detectable under light microscopy, in contrast to the report by Howard et al. [57], but were detectable under fluorescent microscopy. Therefore, when cancer cells are made to overexpress marker proteins, their in vivo behavior may alter and care should be taken to preserve their robustness.

In order to select variants of the NCI-H460 cell line with increased in vivo metastatic potential, Liu et al. [85] used endobronchial implantation followed by isolation of cells from metastatic lesions in the mediastinal lymph nodes, followed by five rounds of consecutive orthotopic reimplantation. This resulted in the isolation of the NCI-H460 variant H460SM. These cells spontaneously metastasize to a variety of soft tissue organs without using tumor pieces as well as to bone when implanted orthotopically in the lungs of nude rats. Eighty-three percent of the cancers metastasized to bone, compared with 8% by the parental cell line. Unfortunately the report [90] does not state when metastasis occurred.

12.3.1.3.2 Intravenous

The first ever reported attempt to develop a lung cancer model of skeletal metastasis via intravenous delivery of lung cancer cells in nude mice involved the injection of the small cell lung cancer (SCLC) cell lines SBC-5, SBC-3, SBC-3/ADM, H69, and H69/VP into the lateral

tail vein of natural killer (NK) cell-depleted SCID mice. Of these cell lines, only SBC-5 cells reproducibly developed hypercalcemia and osteolytic bone metastases [88]. Initial lesions were detected at day 28 and 100% of animals had osteolytic lesions by day 35 post-injection. Sites of metastatic lesion formation included vertebrae, hindlimbs, pelvis, and scapulae. The mice also developed metastasis to the lungs, liver, kidney, and lymph nodes. In this study, SBC-5 cells expressed high levels of PTHrP. Subsequently, it was shown that intravenous delivery of anti-PTHrP antibody significantly diminished the development of osteolytic bone metastasis and of concomitant hypercalcemia in SBC-5-injected mice, but had no effect on the metastasis to other visceral organs [87]. Furthermore, a recent study utilizing this model system indicated that induction of murine osteoclast apoptosis with reveromycin A resulted in a significant decrease in the formation of bone lesions, but had no effect on the formation of visceral metastasis (Fig. 12.1) [90]. SBC-5 intravenous tail injection model can provide insights into the molecular mechanisms of the interaction between SCLC cells and cellular elements of the bone microenvironment, as well as provide a useful preclinical system to assess the efficacy of novel therapeutics on colonization and growth of SCLC in bone.

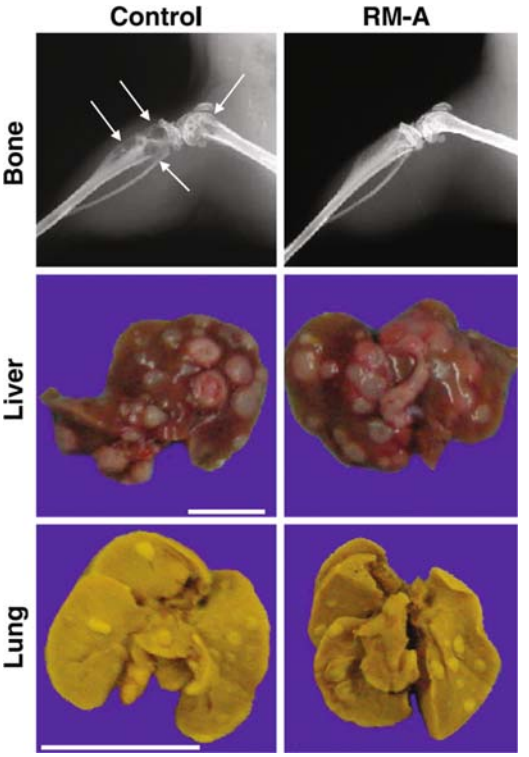

Figure 12.1. Bone-specific anti-metastatic effect by reveromycin A. SBC-5 lung cancer cells (1×10^6 per mouse) were i.v. inoculated into NK cell-depleted SCID mice on day 0. The mice were i.p. administered daily with or without reveromycin A (10 mg/kg) from day 7. Bone metastasis was assessed by X-ray photography on day 35. *Arrows* indicate osteolytic lesions. Comparing treated to control for bone versus liver and lung, one can see the bone-specific effects of reveromycin A as measured by osteolytic lung lesions in bone. Other tissues had no difference in the number of metastases. (Reproduced from Muguruma et al. [90] with permission from the American Association for Cancer Research.)

12.3.1.3.3 Intracardiac

A bone metastasis model was developed by injecting human HARA lung squamous cancer cells into the left cardiac ventricle of nude mice. These cells strongly express and secrete PTHrP. Bone metastases appeared at 4 and 8 weeks after inoculation, but were reduced when the mice were treated with an anti-PTHrP antibody. Increased serum calcium levels were found in mice with bone metastases at 8 weeks after inoculation [60]. When HARA cells were co-cultured with neonatal mouse calvariae, several genes were upregulated, including PTHrP and ezrin, a molecule concentrated in cell surface projections and linked to the spread of many cancers. At sites of bone metastasis, PTHrP and ezrin were upregulated in HARA cells. TGF-β1, which is prevalent in bone marrow, also upregulated ezrin expression [25].

12.3.1.3.4 Intrafemoral/Intratibial

The A549 NSCLC cell line is capable of forming mixed osteoblastic/osteolytic (osteosclerotic) lesions when injected intratibially in nude mice. Treatment with recombinant RANK–Fc significantly reduced intratibial tumor size at 8 weeks following injection (~11,000 mm^3 vs. 700 mm^3). When noggin overexpression was combined with RANK–Fc treatment, the intratibial tumor volumes approached those of sham controls [33]. Therefore, the intratibial injection of A549 cells is an adequate model to investigate

the final stages of the metastatic cascade and allows modeling therapeutic efficacy in well-established bone lesions.

12.3.1.3.5 SCID-hu Models

A major drawback of the xenograft model systems is that they induce human cancer cell metastasis in non-human target organs, including bone. SCID-hu chimeric models, on the other hand, have begun to address the problem of cancer cell: host target tissue disparity. In one such study, samples of human fetal femur and tibia (HFBM) were subcutaneously implanted into SCID mice, subjected to sublethal whole-body irradiation, followed by the injection of SCLC via the lateral tail vein [124]. The number of osteolytic tumors was significantly higher in grafts injected for shorter periods of time. For example, the cell line N4BM colonized HFBM in 65% of mice carrying implants for 3–6 weeks, but only occurred in 8% of the mice that carried the implants for 6–8 weeks. The model appears to be specific for human bone grafts, inasmuch as no metastases were detected in mouse bone [124]. The SCID-hu model is excellent for the study of metastases in vivo, but has the following defects: (1) the need for steps to acquire human bone fragments, (2) the length of time for the engraftment to take, (3) murine vascular elements are part of the established graft, and (4) the relatively slow rate of colonization by the cancer cells. These defects notwithstanding, the model constitutes a significant step forward for the study of the initial interactions between cancer cells and the human bone microenvironment.

12.3.1.4 Transgenic

The number of transgenic models of lung cancer that metastasize to bone are few in number. Meuwissen et al. [86] utilized the frequent inactivation of trp53 and Rb in lung cancer to develop conditional knockouts that developed lung cancers that most resembled SCLC, complete with associated neuroendocrine components. They metastasized, however, only to the liver. C-raf-overexpressing transgenics produced multifocal lung lesions that closely resemble lung adenocarcinoma, but there were no metastases because the primary lesions remained microscopic in size [119]. In another approach, transgenic mice overexpressing 15-lipoxyoxygenase (15-LO) in endothelial cells under the control of the endothelin-1 promoter were injected with Lewis Lung Carcinoma (LLC) cells [49]. The 15-LO transgenic mice had longer survival times and significantly reduced lung metastases.

12.4 Prostate Cancer

PCa is the second leading cause of cancer deaths in American men. In 2007 over 218,000 cases of new PCa in American men were estimated to have occurred and over 27,000 deaths [66]. Overall, 68% of PCa patients will have evidence of disease in bone marrow, making PCa the second cancer most likely to involve bone [118]. It has been estimated that close to 90% of *advanced* PCa metastasize to bone, where myelosuppression or replacement accounts for most PCa deaths [9]. As PCa progresses, hormonal ablation therapy becomes the main treatment. Hormone therapy temporarily slows PCa growth, but eventually PCa becomes refractory to hormone treatment. Bone lesions, most commonly osteoblastic in nature, then result.

12.4.1 Models for the Study of Prostate Cancer Metastasis to Bone

12.4.1.1 Spontaneous

Mice rarely develop spontaneous prostate carcinoma [50]. Therefore, murine models of spontaneous PCa do not exist and murine models rely instead on chemical mutagenesis or transgenic mice. Spontaneous prostate adenocarcinomas are somewhat more common for rats [108].

The Lobund–Wistar (L–W) rat was derived from germfree inbred Wistar strain rats [72]. The seminal vesicle was the initial site of tumor development [55], with additional early carcinomas found in the anterior dorsolateral lobes of the prostate [19, 110, 130]. The initial tumors are androgen-sensitive and spontaneously become refractory, spreading to the lungs, and to the

peritoneal cavity, subsequently invading visceral organs. They do not metastasize spontaneously to bone [111]. However, the Pollard prostate adenocarcinoma (PA) PA-III cell line, which was derived from the L–W rat with spontaneous hormone-refractory PCa, when transplanted adjacent to the rat skeleton, produces an osteoblastic and osteolytic reaction at the site [78].

The Dunning R3327 cell line was also derived from a spontaneous tumor in a male Copenhagen rat, derived from an inbred rat strain and identified first by W.F. Dunning in 1961. Although no metastases were identified in the original Dunning R3327 tumor, in vivo and in vitro passage of the Dunning R3327 cell line has generated sublines with varying androgen-sensitivity and metastatic ability [61].

12.4.1.2 Syngeneic

The MAT-Ly-Lu cell line, a subline of the Dunning R3327 model selected by isolation and serial passage of Dunning R3327 metastases, yields lymph node and lung metastases with very high frequency, when injected intracardially or intravenously into male rats. Inoculation of androgen-insensitive MAT-Ly-Lu cells into the left ventricle of the heart of Copenhagen male rats results in osteoblastic and osteolytic metastases to lumbar vertebrae and hindleg paralysis [11, 48]. Metastatic MAT-Ly-Lu sublines adhere to bone marrow-derived stromal and endothelial cells. This supports the role cell adhesion molecules play in the PCa metastasis to bone [48]. uPA-overexpressing MAT-Ly-Lu cells develop hindlimb paralysis more quickly than controls, and lead to widespread metastases in the ribs, scapula, and femora. Urokinase overproduction leads to greater osteoblast activity [3], whereas inoculation of PTHrP-overexpressing cells results in skeletal metastases with increased osteoclast activity, explaning in part the osteoblastic nature of these metastases. Intravenous injection of MAT-Ly-Lu cells into the tail veins of Copenhagen rats with occlusion of the inferior vena cava results in metastasis in the lumbar vertebrae as well as hindlimb paralysis. This is associated with a transient increase in serum calcium levels, as well as osteoblastic and osteoclastic responses in the lumbar spine, visible microscopically within 4 days. The bisphosphonate, dichloro methylene bisphosphonic acid, suppresses the metastatic potential of the MAT-Ly-Lu cells and delays hindlimb paralysis [41].

The PA-III cell line is one of four cell lines that develops metastases spontaneously in L–W rats. When transplanted to the calvarium or scapula of L–W rats, there is an osteoblastic reaction and a local tumor develops in rat bone. For this to occur, disruption of the local periosteum with the inoculating needle is required [109]. The IGF-I receptor, present on the surface of PA-III cells, mediates the mitogenic effects of IGF-I, IGF-II, and insulin. Conditioned medium from osteoblasts stimulates proliferation of PA-III cells. This suggests that osteoblast-derived IGF may have a major role in promoting the establishment of the PA-III tumor on the rat skeleton [112].

12.4.1.3 Xenograft

12.4.1.3.1 Orthotopic Injection

The LNCaP cell line, marginally tumorigenic PCa cells derived from a human lymph node metastasis [56], do not form bone metastases when inoculated orthotopically into SCID mice. Injection of LNCaP cells into the prostate of SCID mice leads to lymph node and pulmonary metastases, but not bone metastases [121]. However, Thalmann et al. [132, 133] developed a spontaneous bone metastatic PCa model using the parental LNCaP cell line with serial passage through hormonally manipulated male mouse hosts. The LNCaP cells acquire both metastatic and androgen-insensitive properties when inoculated with bone stromal cells in intact athymic nude mice followed by castration and recovery. After several rounds, human cancer cells then can be isolated from the mouse hosts. C4-2, an androgen-independent and bone-metastasizing subline was derived from the parental LNCaP. When C4-2 was injected into the dorsolateral lobe of the prostate, the cancer cells metastasized, eventually yielding the bone-derived sublines, C4-2B2-B5. Chromosome analysis showed that these C4-2 bone-metastasis-derived sublines were of human origin and contained marker chromosomes of the original LNCaP isolated and used to initiate

the study. Bone metastases were osteosclerotic, and immunohistochemistry indicated both the presence of prostate-specific antigen (PSA) and androgen receptor (AR). Additionally, the spontaneous bone metastatic lines C4-2, C42B2-B5 had faster growth rates in vitro and were more invasive than parental LNCaP. Others have generated a number of LNCaP, PC-3, and DU145 variants as reviewed by Sobel and Sadar [126]. Bone-metastatic variants of PC-3 were isolated using multiple cycles of orthotopic injections [107]. However, these cells metastasize predominantly to the mandible and are uniformly osteolytic. As such, they do not represent the most common pathology of human PCa. They are more aggressive than the PC-3M variants derived by Wang and Stearns [139]. Reproducible metastasis to bone from LNCaP and Du145 variants from other investigators has not been as successful. Therefore, the spontaneous bone-metastatic phenotype of the Thalmann et al. clones [136] constitutes an important tool to study bone colonization and therapeutic responses of human PCa cells.

Orthotopic injection of PC-3 and DU145 cells into the NOD-SCID mouse, a cross between the SCID and NOD mouse strains characterized by a lack of functional antigen-presenting cells and NK cells, yields metastases to a variety of soft tissues, but not to bone. However, fragments of a tumor from a subcutaneous xenograft, when implanted orthotopically (SOI) in the prostate of nude mice, produced extensive skeletal metastases, including lesions in the skull, ribs, pelvis, femur, and tibia, as well as metastases to many soft tissues [151].

More recently, an orthotopic model of metastatic PCa has been developed in NOD-SCID mice grafted with human PCa tissue fragments [140]. Regrafting in the anterior mouse prostate led to lymph node metastases, from which the PCa1-met cell line was derived. After 2 weeks, the mice developed multi-organ metastases to soft tissues and bone. The micrometastases of human origin in the bone marrow of the mouse femur were confirmed with a predominant osteolytic phenotype. This represents a new approach to establishing a PCa cell line that metastasizes to bone with high efficiency, in contrast to previous attempts [128].

12.4.1.3.2 Intracardiac Injection

Recently, our group has studied how turnover of host bone modulates colonization of C4-2 cells that have been injected intracardially, with the objective of decreasing latency [133] but increasing bone colonization in the SCID/beige mouse. We hypothesized that because bone turnover rates of SCID mice are low, osteoblastic PCa cells colonize their bones poorly. To increase bone turnover, we tail-suspended them for 3 weeks so as to underweight the limbs and induce bone resorption. In other experiments, the mice were intermittently dosed with PTH(1-34). In both formats PCa cells were injected intracardially at the onset of mechanical overload, or 2 days following the last PTH(1-34) injection. Eight weeks post-C4-2 injection 30% of mice that had received PTH(1-34) pre-treatment were positive for PCa cells (PSA, Pan-cytokeratin) in the proximal femur (Fig. 12.2). Surprisingly, 70% of mice receiving an additional week of PTH(1-34) intermittent dosing following C4-2 inoculation were positive for PCa cells in the proximal femur. In the mice, PSA and pan-cytokeratin-positive cells are localized to the marrow and trabeculae centers of the bone (Fig. 12.2).

Consistent with previous observations regarding the ability of PC-3 cells to colonize bone following intracardial injection [147], Kalikin et al. [68] injected luciferase-tagged PC-3 cells intracardially, and were able to quantify tumor growth in teeth, lumbar spine, and hindlimbs quantitatively, and follow progression longitudinally. The experimental design also allowed comparison of growth rates in young and old male mouse hosts. PC-3 cells grew more aggressively in young mice. This suggests an age-related change in the bone microenvironment, probably a function of bone turnover. In a follow-up publication these authors [122] demonstrated that intermittent PTH(1-34) treatment increased bone colonization over saline controls, but that co-administration of the bisphosphonate zolendronate reduced the PTH(1-34)-induced effect. The increase in bone colonization can therefore be attributed to osteoclast activity, but its mechanism is not known. Because several bone-colonizing tumors express PTHrP, similar mechanisms

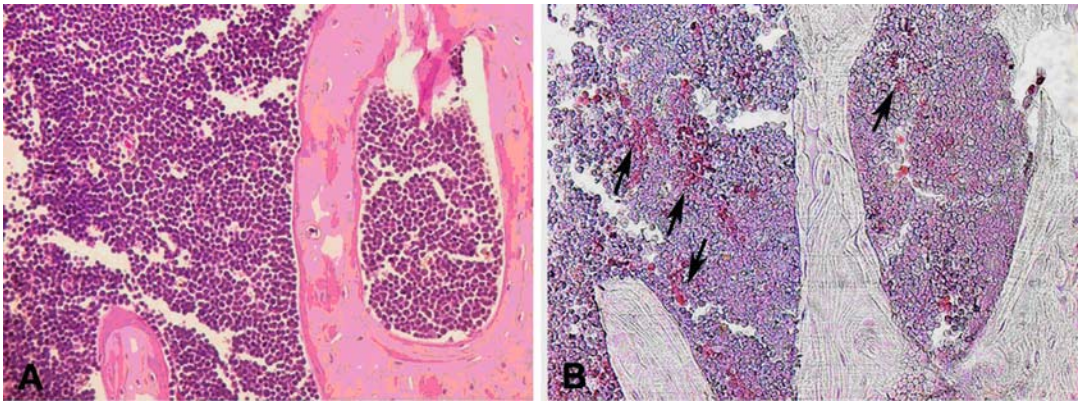

Figure 12.2. Histological examination of proximal femurs from PSA-positive mice injected intracardially with the androgen-insensitive, highly metastatic LNCaP subline, C4-2, following 3 weeks daily intermittent PTH. (**A**) H & E (**B**) Serial section of proximal femur stained with antibody to human PSA 8 weeks post-injection of cells. *Arrows* indicate nests, not single cells, of PCa in the proximal femurs of SCID/bg mice.

may be involved in generating the mixed sclerotic phenotype. Inasmuch as these mouse models permit monitoring bone turnover markers by sampling serum specimens they may serve to correlate tumor colonization with histological data.

12.4.1.3.3 Intravenous Injection

Several studies have shown that intravenous injection of PC-3 cells into the tail vein of male athymic nude mice with occlusion of the inferior vena cava results in development of bone lesions [125,142]. Another PC-3 subline, PC-3.MM2, derived from a liver metastasis produced by a parental PC-3 tumor in the spleen of nude mice, formed bone marrow metastases after tail vein injection. Metastasis was suppressed significantly by treatment with the immunocytokine huKS/4-IL2, directed against a cell adhesion molecule expressed by metastatic human PCa [29]. All of these lesions are osteolytic and represent a small subset of human PCa; however, their aggressive nature and fast growth make possible preclinical studies to determine the effect of bone turnover on osteolytic disease.

12.4.1.3.4 Intrafemoral/Intratibial Injection

Significant differences exist among different immunocompromised murine hosts in regards to the ability of cancer cells to expand beyond dormant single-cell foci in vivo and to colonize bone. For example, LNCaP cells failed to grow in some athymic hosts, whereas C4-2 cells metastasized to the ilia and femora. C4-2B4 cells induced osteosclerotic bone lesions 45% of the time in athymic mice when injected intrafemorally, but not intrailially. PC-3 cells injected intrafemorally in athymic mice produced osteolytic lesions at a rate of 100%. In SCID/bg mice, however, the results were different: LNCaP (75%), C4-2 (100%), and C4-2B4 (100%) cells, when injected intrafemorally in SCID/bg mice, produced osteosclerotic tumors (Fig. 12.3) and secreted PSA. However, LNCaP tumors in marrow spaces were limited to the confines of the marrow while C4-2 and C4-2B4 lines expanded beyond the marrow cavity [147]. Soos et al. [127], using the nude beige mouse, showed that LNCaP cells produced intrafemoral lesions with an osteosclerotic phenotype in 75% of direct injections. Results similar to those obtained by Wu et al. [147] were found for PC-3 cells (Fig. 12.3). These findings were confirmed later with human PCa cell lines that were injected intratibially into athymic mouse hosts [35]. These models are useful to study the biology of advanced PCa lesions in bone and to test novel therapeutic agents to inhibit or eradicate osteosclerotic lesions. These models do not, however, allow study of the metastatic process which requires conversion from dormancy to permit bone colonization [148].

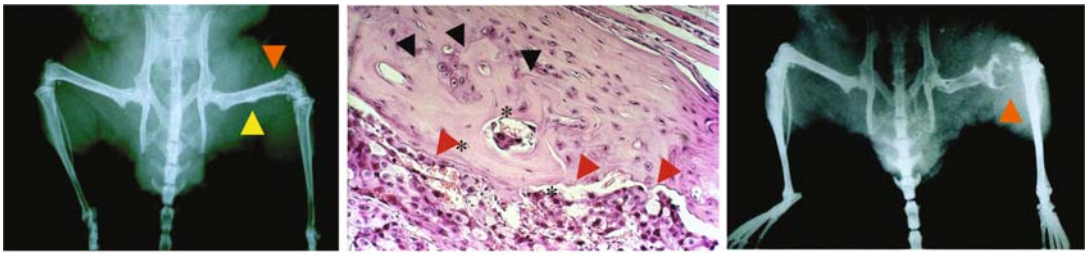

Figure 12.3. Intraosseous injection of osteosclerotic and osteolytic prostate cancer cells intrafemorally in SCID/bg male mice. C4-2 cells produce areas of osteosclerotic lesion in femur marrow spaces, as seen at arrowheads (*left*). Mixed lesions are noted in H & E sections for C4-2 tumors shown in radiographs (*center*). *Reversal lines* denoting newly deposited osteoid are present (*Black arrowheads*). *Red arrowheads* indicate direct contact of tumor cells with osteoid surface with evidence of sculpting. PC-3 cells, by contrast (*right*), have a profound osteolytic phenotype and completely degrade bone (*Arrowhead*). This type of lesion represents less than 5% of all prostate carcinomas. (Modified from Wu et al. [147] with permission.)

12.4.1.3.5 SCID-hu Mouse Models

Nemeth et al. [98] implanted fragments of human fetal bone, lung, or intestine subcutaneously into SCID mice and, when the grafts were established, PCa cells, PC-3, were injected intravenously. The PC-3 cells colonized the implanted human bone fragments in 5 of 19 mice, with the usual osteolytic response. LNCaP cells failed to colonize human fetal bone grafts. However, when PC-3, Du145, and LNCaP were engrafted into human fetal bones, large tumors resulted. This model is now used in preclinical studies to evaluate new treatments for bone metastases of PCa [10]. Cancer cells have also been injected into SCID-hu bone engraftments to establish models of PCa interaction with bone [134].

12.4.1.4 Transgenic

In the TRAMP model, transgenic mice were generated by using the prostate-specific rat probasin promoter to drive expression of the simian virus 40 large T-antigen-coding region, resulting in the expression of both the large and small T-antigen. The PB-SV40 T-antigen (PB-Tag) was shown to be restricted spatially to the ventral and dorsolateral prostate, where prostate tumors develop as early as 10 weeks, and invasive carcinoma has been observed as early as 18 weeks in these mice [46]. TRAMP mice maintained as [TRAMP × C57BL/6]F1 or [TRAMP × FVB]F1 offspring demonstrated frequent metastases to lung and lymph nodes and occasional metastasis to bone. Hindlimb paraplegia was observed in a 22-week-old [TRAMP × FVB]F1 mouse, and histological examination of decalcified sections of the spine revealed metastatic tumor cells throughout the spinal canal. The inner surfaces of the vertebrae were composed of newly formed woven bone; this indicates an osteoblastic response. However, this was not true for the [TRAMP × C57BL/6]F1 mouse. The frequency of bone metastasis may therefore be a function of a murine strain-specific response [42].

Recently, bigenic TRAMP-Luc mice were generated and obtained as [C57BL/6 TRAMP × FVB sPSA-Luc] F1 offspring to monitor local prostatic tumor growth and metastasis [60]. This model allows tracking the AR activity in TRAMP tumors, as well as noninvasive imaging of bone and soft-tissue PCa metastases [59]. While other transgenics, e.g., PSMA [16, 26, 146] and knockout mice, e.g., PTEN, NKX3.1 [1, 9, 38, 73], have developed PCa that, in some instances, progressed to androgen independence, in most the cancer did not metastasize to bone.

12.5 Renal Cancer

It has been estimated that 51,190 patients were diagnosed with carcinomas of the renal pelvis and kidney, and 12,890 renal cancer deaths occurred in 2007 [66]. Approximately 85% of all kidney cancers are renal cell carcinoma (RCC) and account for about 43,500 cases in 2007 [116]. Renal carcinomas arise

most frequently from cells of the proximal convoluted tubules. Approximately 25%–30% of patients diagnosed with renal carcinoma present with evidence of locally advanced cancer and metastasis to other organs such as lungs, bone, liver, and brain. About 35% of renal carcinoma patients will develop bone metastasis resulting in spinal cord compression and pathologic fractures. Renal carcinomas are frequently refractory to chemotherapy and radiation, therefore, treatment options are limited to palliative therapy.

12.5.1 Models for the Study of Renal Cell Cancer Metastasis to Bone

12.5.1.1 Spontaneous

RCC occurs spontaneously in rats and mice, but rarely [50,123], and does not metastasize to bone. The Eker rat model of RCC is the result of a spontaneously arising neoplasm that leads to carcinoma and invasion of the surrounding renal tissue [32]. Eker renal tumors have been described as simple cysts, papillary cystadenomas, solid eosinophil adenomas, or solid basophile tubular adenomas. They often involve the entire kidney, yet RCC tumors in Eker rats are nonmetastatic [32].

Another spontaneous model of RCC is the Wistar–Lewis rat model [144]. This tumor is maintained by surgical implantation in the flank of syngeneic rats and takes about 3 weeks to develop. It does not metastasize [145].

Another spontaneously developing model of RCC is the Renca murine model, a tumor that arose spontaneously in a Balb/c mouse [58, 93]. Renca can be cultured or passaged as a xenograft by subcapsular renal injection and/or peritoneal injection in Balb/c mice; the resulting tumor is a granular cell-type adenocarcinoma. Following subcapsular implantation, metastases develop in lymph nodes, lung, liver, and peritoneum [54]. Because Renca mice survive only about 5 weeks after tumor development, they are a good model for chemotherapeutic studies. However, a major limitation is the lack of metastasis to bone.

12.5.1.2 Xenograft

Human RCCs have been transplanted successfully into nude mice. Their morphology, growth, and chromosomal profiles are those of renal carcinoma patients [18, 77, 104]. Metastatic potential, however, may be compromised [77]. Portions of human renal carcinosarcoma have been transplanted into athymic nude mice. These transplants differentiate into bone, cartilage, and skeletal and smooth muscle, but do not transplanted successfully metastasize [14, 104].

12.5.1.2.1 Orthotopic Injection

Orthotopic injections of renal carcinomas in mice have not led to bone metastasis. Zisman et al. developed an orthotopic RCC tumor model in SCID mice from human metastatic chromophil (papillary) renal cell carcinoma (cRCC) tumor cells that express the tumor-associated antigen carbonic anhydrase type 9 (CA IX) [155]. This model metastasized only to liver and lung, but allowed studying aggressive, high-grade cRCC, and developing CA-IX-related therapies. Human RCC injected into the renal subcapsule of nude mice led to more extensive metastases to soft tissues, demonstrating the significance and utility of orthotopic implantation [34, 94, 95], as compared to subcutaneous.

12.5.1.2.2 Intrafemoral/Intratibial Injection

The human cell line RBM1 was isolated from a patient with RCC [142] and is currently the only model suitable for studies regarding the malignant spread of RCC to bone. The RBM1 cell line displays a trisomy of chromosome 7, and high levels of EGF-R expression and responsiveness. Upon intratibial injection, the RBM1 cell line induces osteolysis in nude mice [142]. In a subsequent study, the authors isolated cells from these lytic lesions, and termed them RBM1-IT4. When these were injected, 86% of the mice developed tumors. Intratibial injection of the transgenic RBM1-IT4 cell line, which expresses the dominant-negative TGF-β type II receptor, inhibits tumor formation and osteolysis. This highlights the significant role played by TGF-β1 in facilitating interactions between RCC cells and the bone microenvironment [76].

Use of a tyrosine kinase inhibitor in combination with Taxol resulted in decreased EGF-R activation, as well as a statistically significant decrease in intratibial tumor take, tumor size, and bone lysis [141]. In another study, the RBM1-IT4 cell line was used to test the

importance of VEGF signaling on the growth of RCC both within the kidney and bone of nude mice [70]. Treatment with the anti VEGF-R tyrosine kinase inhibitor TSU-68 five days after tumor cell implantation significantly inhibited tumor growth in the kidney, but failed to inhibit tumor growth when injected into the tibia.

12.6 Multiple Myeloma

Multiple myeloma (MM) is a malignancy of the plasma cells in the bone marrow, causing osteolytic lesions associated with pathologic fractures, spinal cord compression, and hypercalcemia. MM is the most common primary neoplasm of the bone and infiltrates the skull, ribs, pelvis, vertebral column, and proximal long bones. An estimated 19,900 new cases of myeloma were diagnosed in 2007, with 10,790 deaths [66]. Eighty percent of MM patients present with essentially osteolytic bone pain and lesions. The lesions are due to factors associated with osteoclastogenesis, such as PTHrP and IL-6, which stimulate osteoclastic activity. Myeloma cells may also trigger an increase in RANK ligand and a decrease in osteoprotegerin in the bone marrow, as well as causing release of the macrophage inflammatory protein to promote bone resorption [2, 43, 44]. Current mouse models are limited in how they reflect the human disease.

12.6.1 Models for the Study of MM Metastasis to Bone

12.6.1.1 Spontaneous

Aging C57BL/KaLwRij mice are susceptible to the development of monoclonal gammopathies [36]. In the bone marrow of C57BL/KaLwRij mice with an excessive homogenous immunoglobulin component in the serum, myeloma cells expand monoclonally, as detected by immunocytochemistry. Furthermore, spontaneous development of MM in C57BL/KaLwRij mice results in characteristic osteolytic lesions and transplantation of these cells to C57BL/KaLwRij hosts recreates the disease precisely [114]. The 5T33 cell line was created in a similar manner from bone marrow aspirates of aged C57BL/KaLwRij mice [39] and, when injected, led to osteolytic destruction of bone. This model has been improved when 5T2MM cells were injected into ovariectomized C57BL/KaLwRij mice. The mice developed paraproteinemia and osteolytic bone lesions two weeks before non-ovariectomized mice, but the bisphosphonate, pamidronate, prevented osteolytic lesions in ovariectomized mice [83]. Injection of a cell line, 5THL, derived from ovariectomized C57BL/KaLwRij mice bearing 5T2MM cells, resulted in much paraproteinemia and osteolytic lesions [84].

12.6.1.2 Syngeneic

The ectopic expression of IL-1 alpha in the IL-6-dependent murine B cell hybridoma B9 gave rise to the B9/BM1 cell line, which in turn expresses high levels of the cell adhesion molecules CD44 standard and of $\alpha_4\beta_1$ integrin [51]. Intravenous injection B9/BM1 cells yielded bone marrow metastases and bone lesions [52]. Because monoclonal antibodies to both CD44 standard and $\alpha_4\beta_1$ integrin decrease the adhesion of B9/BM1 cells to primary cultures of murine bone marrow endothelial cells [101], this model may be useful for testing the biological determinants of myeloma metastasis.

12.6.1.3 Xenograft

12.6.1.3.1 Intravenous Injection

The ARH-77 cell line was established from the peripheral blood of a female Caucasian patient suffering from IgG plasma cell leukemia [12]. The cell line is an EBV-transformed B lymphoblastoid cell line [30]. The ARH-77 cell line has been used extensively in xenograft studies of MM. In one such study, 100% of mice developed hypercalcemia (mean of 5 days postparaplegia) and hindlimb paralysis (28–35 days post injection) [4]. In addition to liver and spleen being involved, ARH-77-bearing hosts develop osteolytic bone lesions and a high number of osteoclasts. The integrin expression profile of ARH-77 is known [136]. Like other MM cell lines, ARH-77 cells are beta-1 and alpha-4 positive. ARH-77-bearing SCID mice have been used to test bisphosphonate [21] and monoclonal antibody [71] in the treatment of MM.

Another commonly used cell line for MM studies is the autocrine IL-6-stimulated human cell line KPMM2 [45]. Because KPMM2 is a free-standing cell line that expresses the MM marker M protein, KPMM2 cells are very useful in MM studies. Subcutaneous injection of KPMM2 causes mice to develop solid, transplantable tumors in 2 weeks, but no metastasis. Mice injected IV with KPMM2 cells develop severe fatigue, loss of balance, weight loss, and hindlimb paralysis in 30–40 days, with their life span not exceeding 43 days [135]. In 37 days the myeloma cells account for over 60% of the marrow cell volume. Other sites of metastasis include abdominal lymph nodes and kidney. SCID mice injected with KPMM2 cells also develop hypercalcemia and striking levels of bone resorption/destruction. Thus, IV injection of KPMM2 cells into nude mice seems to closely approximate the natural disease course of MM in humans.

12.6.1.3.2 SCID-hu Mouse Models

The SCID-hu model is probably the best model for MM, inasmuch as normal SCID mice do not allow engraftments of human MM. In addition to their effect on murine bone, ARH-77 MM cells also home to human bone grafts in vivo. Following the grafting and irradiation of femur and tibial fragments from 19–23-week-old fetuses into both sides of SCID mice, injection of ARH-77 cells into the fetal bone implant marrow cavity leads to infiltration within 4 weeks. Moreover, the ARH-77 cells homed and infiltrated the right fetal bone implants by 12 weeks post-injection [137], but did not infiltrate the murine bone. The model can utilize other MM cell lines, including OCI-My5, IM-9, HS-Sultan, and RMPI-8226.

In another model, IL-6-dependent human MM cells, INA-6, were engrafted into SCID mice with implants of human fetal bone chips. Soluble IL-6 receptors (shuIL-6R) were detected in engrafted, but not in control SCID mice. Because MM cells require human bone marrow for growth and survival, this model constitutes a reproducible system for human bone microenvironment-dependent MM growth which can be used to test anti-MM agents [131].

12.6.1.4 Transgenic

The transgenic models of plasmacytoma, tumors formed by collections of mycloma cells, [129,117] display virtually no bone involvement, but have provided important insights into the oncogenic pathways to malignancy [36].

By crossing Myc and Bcl-X_L transgenic mice, Cheung et al. [17] obtained a transgenic model that displayed rapid onset, high penetrance of human plasmacytoma, extensive bone marrow involvement resulting in "wall to wall" tumors in bone, with some mice displaying osteolytic lesions and pathological fractures. The authors speculate that crossing the two types of transgenic mice increased expression of genes associated with MM progression (Abl, FGFR3, ras, wnt, etc.). It also increased transcription factors associated with plasma cell commitment (Blimp-1, XBP-1, IRF-4), as well as chemokine receptors such as CXCR-4, that direct plasma cells from lymph nodes to bone marrow. An increase in adhesion molecules that stimulate plasma cell homing to bone marrow, and of mediators of bone cell destruction, will provide a true transgenic model of MM.

12.7 Melanoma

Malignant melanoma, a cancer of melanocytes, represents only 3% of skin cancers, but accounts for 65% of skin cancer deaths. A total of 59,940 new cases of melanoma and 8,110 deaths were predicted for 2007 [66]. Melanoma spreads to lymph nodes and adjacent skin, then to lung, liver, brain, bone marrow, and intestine [24]. In 5–10% of cases, metastatic melanoma is detected in the absence of a detectable primary tumor, which most likely has regressed [5, 8, 15]

12.7.1 Models for the Study of Melanoma Metastasis to Bone

12.7.1.1 Spontaneous

Spontaneous melanomas in mice are rare because there are few melanocytes in mouse dermis. The highest concentration of melanocytes is in or near the tail and in the ears. Con-

sequently, most spontaneous melanomas are thought to originate in poorly differentiated tumors like basal cell carcinoma. The overall incidence of melanoma, including amelanotic melanomas, may be near 0.5% with only 0.1% being malignant [106]. The incidence of metastasis to lymph nodes, lungs, and liver is low.

12.7.1.2 Syngeneic

When GFP-transfected B16 melanoma cells were injected into the tail vein of female C57BL/6 mice, metastases arose in the brain, lung, pleural membrane, liver, kidney, adrenal gland, lymph nodes, skeleton, muscle, and skin. The tumor had colonized bone and bone marrow in the vertebrae, pelvis, femur, tibia, humerus, and the flat bones of the skull and scapula (Fig. 12.4) [151]. In another study, intravenous injection of B16/F10 melanoma cells in C57BL/6 mice led to metastases in lung, mandible, tibia, and femur [149]. Injection of B16 melanoma cells into the left cardiac ventricle of female C57BL/6 mice caused the tumor to colonize the bone and bone marrow of the axial skeleton, the cranium, mandible, maxilla, vertebral bodies, and pelvis. Metastases were also found in the proximal large bones of the extremities, the distal ends of the ribs, and the thoracic and lumbar regions of the spine. In a separate study, intracardial injection of B16 melanoma cells into C57BL/6 mice produced visceral and bony metastases 10 days after injection (Fig. 12.5). Seventy percent of the bones had widespread tumor growth, with trabecular bone loss correlating with MRI analysis, even though X-ray radiography did not distinguish between tumor and non-tumor-bearing animals [40, 143].

TIMP-3 plays an important role in regulating melanoma metastasis. When C57/B16 B16F10 melanoma cells were injected intracardially into timp-$3^{-/-}$ mice, there was a 14-fold increase in metastatic load in the kidney, a fivefold increase in metastatic load in the liver, and a twofold increase in the lung, compared to controls. Melanotic metastases occurred in both wild-type and timp-$3^{-/-}$ mice, but the metastases were nearly twice as great in the timp-$3^{-/-}$ mice, who also had more extensive bone degradation in the trabecular region [22].

12.7.1.3 Xenograft

12.7.1.3.1 Intravenous Injection

Injection of human melanoma cells has not produced the wealth of metastases observed with other cancer-derived human cells. Byers et al. [13] have shown that some of these failures and differences in the scope of human melanoma cell metastases are due to immunocompromised host strain differences. Konniksen et al. [74] showed that FEMX-1 human melanoma cells killed nude rats because metastases grew rapidly in the lungs. Yet, when the same cells were injected into the tail vein, no lung metastases resulted. Evidently host and organ factors account for preferential colonization, but this does not entirely explain the poor colonization of murine bone by human malignant

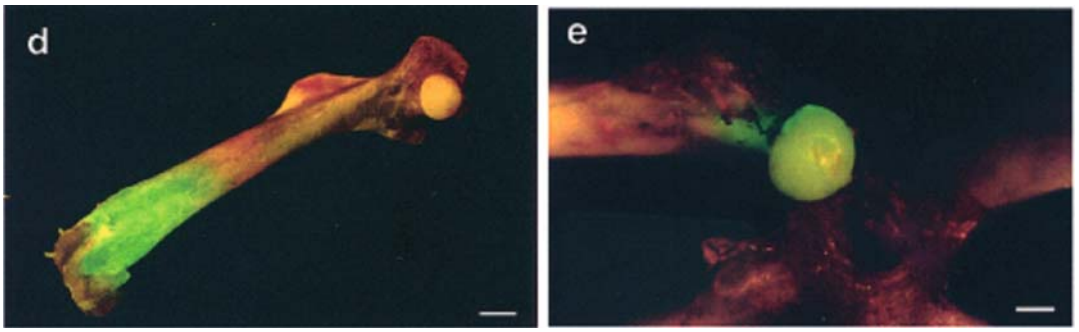

Figure 12.4. GFP-labeled B16F0 melanoma cells were injected via tail vein and sacrificed 3 weeks later. Fluorescent imaging of skeleton revealed metastases in multiple skeletal locations including the distal end of the femur (**d**) and the femur heads (**e**) of some animals. These techniques allow monitoring of smaller tumor burdens than previous techniques. (Modified and reproduced from Yang et al. [152] with permission from the American Association for Cancer Research.)

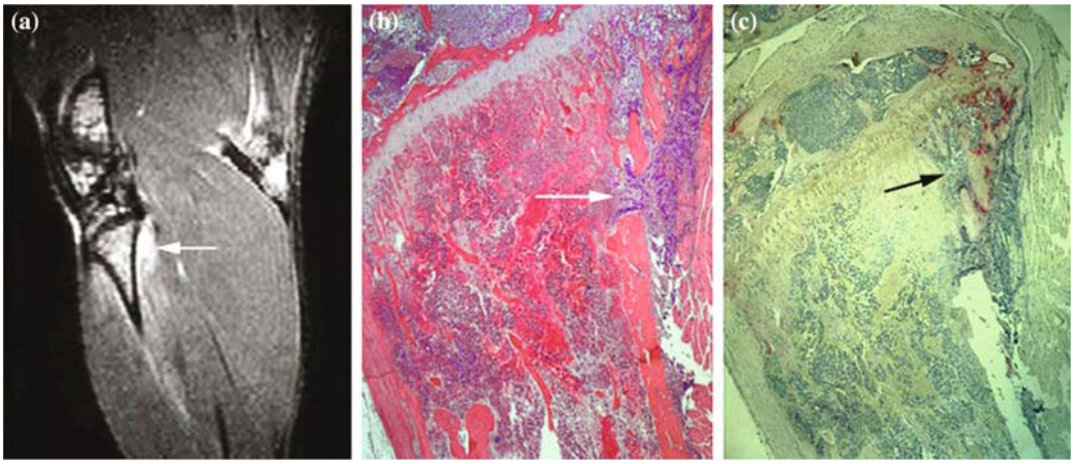

Figure 12.5. (**a**) T2-weighted image through the tibia of a B16 melanoma tumor-bearing mouse. Corresponding histology through the tumor is shown by (**b**) H & E and (**c**) TRAP stain, identifying osteoclasts. The tumor is seen extending into the soft tissue without cortical destruction on both the MRI and corresponding histology slides (*arrows*). The lesion extending outside the cortex measures 150 μm in diameter. (Reproduced from Gauvain et al. [40] with kind permission from Springer Science and Business Media.)

melanoma. MeWo malignant melanoma cells, pigmented when isolated from the patient, give rise to primarily amelanotic lung metastases when injected through the tail vein (Fig. 12.5). Ishikawa et al. [62] have developed a model for studying the metastatic colonization by human melanoma cells of bone and other organs by selecting for MeWo cells that are resistant to wheat germ agglutinin. These cells, named 70-W, colonize and grow in skin, brain, bone marrow, gut mesentery, and muscle, with all metastases melanotic. The composition of cell surface glycoproteins therefore seems critical for metastasis and colonization. This may also be true in lymph node colonization.

12.7.1.3.2 Intracardiac Injection

LOX human melanoma cells, when injected in the left ventricle of nude rats, produce hind-leg paralysis in all animals after two weeks. Metastases are present in the lumbar spine, long bones, and occasionally the skull [75]. In a separate nude mouse model, the role of TGF-β in melanoma-induced osteolysis was examined by intracardially injecting 1205Lu melanoma cells that overexpress Smad 7, an inhibitor of TGF-β/Smad-signaling, in athymic nude mice. 1205Lu melanoma cells exhibit high autonomous ligand-induced constitutive TGF-β/Smad signaling and are highly invasive in Matrigel. 1205Lu-Smad7 cells injected in the left cardiac ventricle of mice induced less osteolysis and longer survival than parental and mock-transfected 1205Lu cells. 1205Lu-Smad 7 bone metastases expressed much lower levels of the connective tissue growth factor, IL-11 and PTHrP, compared to controls. Further, activation of IL-11, CXCR4, osteopontin (OPN), and PTHrP was reduced. Inasmuch as TGF-β increases the steady-state levels of mRNAs, TGF-β must be vital to melanoma bone metastasis and osteolysis [64].

12.7.1.3.3 Intrafemoral/Intratibial Injection

As with other cancers that metastasize to bone, LOX human melanoma cells will grow tumors in bone when directly injected, producing primarily osteolytic lesions with radiographic properties that are similar to those observed in patients with bone metastases from melanoma. The bone-engrafted melanoma cancers are resistant to doxorubicin, but sensitive to radioactive strontium and to an alkylating agent, mitozolomide. Therefore, intraosseous delivery of melanoma can serve as a preclinical screen to monitor therapeutic responsiveness at reduced cost than using nude rats and at a greater rate.

12.7.1.3.4 SCID-hu Mouse Models

First steps have been taken to ascertain whether melanoma cells interact directly with the bone microenvironment in aggressive melanoma [128]. This study utilized a novel bilayer soft agarose assay where hBMSC cells are seeded in the bottom layer, with the melanoma cells in the top layer. hBMSC stimulated the soft agarose cocony forming potential of the melanoma cells up to 13-fold, demonstrating the potential significance of signaling from human bone marrow stromal cells during melanoma colonization of human bone marrow.

12.8 Concluding Remarks

Clinically relevant animal models are needed to understand fully the nature of tumor formation and the growth process and to improve the therapeutic response. Early xenograft models made possible and determining preclinical testing for determining the efficacy of chemotherapeutics. They allowed monitoring the consequences of chemotherapeutic failure and devising alternative strategies to combat "resistant" disease. Because many metastases are aggressive and resistant to therapy, models of cancer metastasis had to be developed. For cancers that metastasize to bone, this is an ongoing task. Getting primary xenografts to metastasize or colonize bone has not been straightforward. Destruction of bone architecture with direct injection of cancer cells provides a means to analyze cancer interaction with bone, but the damage from injection may provide an unnatural foothold for cancers. Intracardiac injection is often effective, but may fail to lead to metastases or yield cells that do not form overt osseous metastases. Yet these cells may be critical for cancer progression, constituting dormant tumor cells. Even though primary tumors release hundreds of thousands of cells into the circulation daily, overt clinical metastases that develop are few in number. Clearly, the cancer cells found in many tissues are viable. The model must therefore address two problems: First, what are the criteria for a significant metastasin to bone? Factors to consider include cancer stem cells, hematopoietic precursors present in primary tumors, the metabolic activity of the targeted bone, and its turnover. Second, what are the critical components for maintaining and awakening dormant cells that metastasize to bone and other tissues? These factors likely contribute to the development of the lethal cancer phenotype involving bone. Keys to maintaining cancer cell dormancy may allow for long-term remission and stable disease.

Available techniques remain crude but are improving. Histology and immunohistochemistry are a mainstay but are giving way to techniques that allow repeated measurements and enhanced resolution. This includes X-ray imaging by radiographs, microCT, and magnetic resonance imaging. Additional techniques comprise GFP- and/or luciferase-transfected cancer cell lines that permit enhanced quantitation and better understanding of how bone responds to cancer cells. These techniques measure only the response by bone, the host tissue, and not the process of metastasis. This remains for future research.

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13.

Hormonal therapies in Breast and Prostate Cancer: Effects on Bone and the Role of Bisphosphonates

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13.1 Introduction

Many patients with cancer are at risk of skeletal-related complications including pathologic fractures and chronic pain because so many tumors metastasize to bone, leading to significant morbidity and mortality. Certain solid tumors have the propensity to metastasize to bone, most commonly breast cancer (BCa) and prostate cancer (PCa). In addition, chemotherapy and hormonal therapies contribute to bone loss and increased incidence of osteoporotic fractures. Medications known as bisphosphonates, commonly used in osteoporosis treatment, are also approved for metastatic bone disease. Recent evidence suggests that these agents may slow the progression of metastatic bone disease and, because they have an anti-tumor effect, help prevent skeletal events. In this chapter, we will review the effects of newer endocrine/hormonal therapies for BCa and PCa and discuss the role of bisphosphonates for both treatment and prevention of skeletal events. Prevention of bone loss due to these treatments will also be considered.

13.2 Hormonal Therapies in Breast and Prostate Cancer

Hormonal deprivation therapy is the mainstay of treatment in BCa and PCa that are hormone-sensitive. Hormone deprivation is first-line therapy, with treatment begun early in the course of the disease, even before the onset of bone metastases. Breast cancer is treated with anti-estrogens or aromatase inhibitors (AIs) when estrogen or progesterone receptors are positive. PCa has no specific markers other than the prostate-specific antigen (PSA), but androgen deprivation therapy (ADT) by means of gonadotropin-releasing hormone agonists (GnRH-agonists) is the primary therapy that is used continuously or intermittently until PCa becomes androgen-independent.

13.2.1 Prostate Cancer

Prostate carcinoma (PCa) is the most common cancer and the second leading cause of cancer death in US men [51]. Men have a 16% chance

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of developing PCa in their lifetime and approximately 1 in 34 will die from it. The median age at diagnosis is 68 years; black men have a 40% higher risk of developing PCa than whites and die from PCa twice as often as white men [76]. The decline in mortality in the past decade can be attributed in part to earlier detection made possible by testing for PSA and by better therapies for local and advanced disease.

13.2.1.1 Use of Gonadotropin-Releasing Hormone Agonists (GnRH-Agonists) in Prostate Cancer

PCa is commonly a disease of older men, with 50% of those over 75 years having other comorbidities. PCa is typically a slowly progressive disease, with many older men living with the consequences of cancer treatment. A major consequence of PCa treatment is hypogonadism. As a result of ADT, testosterone and estrogen drop to castrate levels. Because testosterone levels decrease as men age, more than half the men over 80 years are hypogonadal even before initiation of GnRH-agonist treatment [31]. Hyogonadism leads to decreased bone density, decreased muscle mass, increased risk of falls, and impaired balance. Because GnRH-agonist therapy leads to an extreme form of hypogonadism, it induces the geriatric syndrome of frailty even in younger men.

Some 70% of PCas are androgen-dependent [63] and thus respond to hormonal ablation therapy, whether by surgical castration (bilateral orchiectomy), or treatment with estrogens or anti-androgens. Since late in the 1980s, GnRH-agonists have been widely used for local and advanced disease. Between 33% and 70% of men with PCa now receive GnRH-agonists as primary therapy [63,69], with anti-androgens to treat localized disease. Often men with a rising PSA are treated with GnRH-agonists after primary therapy with surgery or radiotherapy, with the widest use in men older than 80 years [65].

13.2.1.2 Neoadjuvant and Adjuvant Therapy with GnRH-Agonists for Prostate Cancer

Neoadjuvant therapy is commonly combined with radiation therapy or surgical radical prostatectomy in men with local or locally advanced disease, but no metastases. Typically, the neoadjuvant ADT is injected 3 months prior to the start of radiotherapy or prostatectomy. A meta-analysis from the Cochrane database concluded that neoadjuvant treatment prior to radical prostatectomy did not improve overall survival, but led to a significant decrease in positive surgical margins and a borderline decrease in disease recurrence [42]. The rationale for use of neoadjuvant therapy prior to radiotherapy is to shrink the tumor [61] and thus the amount of radiation required. When neoadjuvant therapy was given prior to radiation treatment, survival was improved in patients with well-differentiated disease. Disease-free and biochemical disease-free survival was also improved [42]. No improvement was seen in disease-specific survival.

Adjuvant GnRH-agonist treatment, when given simultaneously with external irradiation, improves local control as well as disease-free and overall survival of patients with locally advanced PCa when compared with androgen deprivation alone [5]. Side effects such as hot flashes, gynecomastia, and bone loss must be considered when these agents are used with adjuvant therapy.

13.2.1.3 Locally Advanced Disease

Men over 80 years, with local recurrence and rising PSA after primary therapy such as prostatectomy or radiation, have been increasingly treated with ADT, but whether this prolongs their survival is still unanswered.

13.2.1.4 Metastatic Disease in PCa

Clinically significant bone metastases occur in 80–90% of advanced PCa patients [51]. The establishment of metastases depends on the bone microenvironment allowing PCa cells to take up residence in bone. Although PCa skeletal metastases are mostly osteoblastic in nature, they also have a bone resorptive component [2]. BCa, although primarily osteolytic, also has an osteoblastic component.

13.2.1.5 Consequences of GnRH-Agonist Treatment

Although treatment with GnRH-agonists has mitigated pain and reduced cancer-related morbidity in patients with advanced-stage disease, it has led to adverse effects such as hot flashes,

cognitive changes, depressed mood, lower bone mineral density (BMD), and unfavorable lipid changes [2].

13.2.1.6 Loss of Bone Density

GnRH-agonists constitute a major risk factor for osteoporosis in older men. Some 2 million men have osteoporosis and 12 million are at risk. Men account for one-third of all hip fractures and have higher 1-year mortality rates after hip fracture than women. The rate of annual bone loss in the healthy male population is between 0.5% and 1.0% [45], whereas men on GnRH-agonist therapy lose 2–3% per year at the hip and spine initially [72] and, after some years, the loss is less but lasts throughout therapy [43].

GnRH-agonist therapy leads to increased bone remodeling. Increased osteoclast activity is reflected by an increase in the C-telopeptide level in the serum and the increase in osteoblast activity by a raised serum level of the amino-terminal propeptide of type I procollagen, P1NP. These markers reflect bone changes fairly soon, often within weeks, whereas bone density changes may take months or years. The rise in remodeling due to GnRH-treatment ultimately leads to bone loss and increased fracture rates [69].

13.2.1.7 Estrogen Loss Due to GnRH-Agonist Treatment

Testosterone as the predominant male hormone plays an important role in bone health, but men also biosynthesize estrogen, although at levels substantially lower than in women. In both men and women, testosterone is converted through aromatization to estrogen in tissues such as the testes, liver, adrenal, and adipose cells. In recent years, both epidemiologic and interventional studies have called attention to the critical role estrogen plays in building and maintaining bone [39]. Because GnRH-agonist therapy also lowers male estrogen to castrate levels, it has an impact on men who receive ADT for PCa.

Medical castration with estrogen, specifically diethylstilbestrol (DES), has historically been the treatment for metastatic PCa. The hip BMD of men who have been surgically castrated with bilateral orchiectomies initially decreases approximately 10% per year, whereas hip BMD decreases only 1% a year in men given estrogen for medical castration [20]. It is not surprising, therefore, that men with PCa who receive estrogen transdermally have higher BMDs [52] and, if given estrogen in the form of oral micronized estradiol, respond with a decrease in bone turnover [74]. However, long-term estrogen trials have not yet been carried out.

13.2.1.8 Fractures and GnRH-Agonists

Several large studies have now confirmed that GnRH-agonist therapy increases the risk of fractures. On the basis of the SEER study [65], 19.4% of men who were receiving GnRH-agonist or had undergone orchidectomy experienced fractures, whereas untreated men with PCa had only a 12.6% incidence of fractures. Moreover, several studies have demonstrated that the longer the treatment duration, the higher the risk of fracture [43,71]. Finally, in a study of medical and pharmacy claims from 16 companies, rates of fracture were significantly increased (7.91 versus 6.55 per 100 person years) in men treated with ADT versus those who were not treated (relative risk 1.21). Age and co-morbidities were independently associated with fracture risk, as was GnRH-agonist treatment [70].

13.2.2 Breast Cancer (BCa)

BCa is the most commonly diagnosed cancer in women. It affects more than 200,000 American women per year and is the second leading cause of cancer-related deaths in women [36]. Endocrine therapy has been a key aspect of how patients with hormone receptor-positive BCa have been managed. For many years, tamoxifen has been the gold standard for first-line endocrine treatment for BCa. Recently, treatment with the AIs letrozole, anastrozole, and exemestane has led to better outcome than tamoxifen in postmenopausal women with early-stage BCa. Als have been used as first-line therapy, as an alternative to tamoxifen, and after completion of 5 years of tamoxifen therapy [3,14,28]. AIs are more effective than tamoxifen in preventing BCa disease recurrence, whether used as first-line therapy or after tamoxifen treatment for early-stage BCa. AIs along with

ovarian suppression is also finding use in the treatment of premenopausal women.

13.2.2.1 Tamoxifen and Bone

Tamoxifen is a selective estrogen receptor modulator. It antagonizes estrogen in breast tissue, but has estrogen agonistic effects at most other estrogen receptors, including bone, uterus, and in lipid metabolism [46,47]. In postmenopausal women with low endogenous estrogen levels, tamoxifen treatment causes bone resorption to decrease, with a small but significant increase in lumbar spine and total hip bone density [58]. At premenopausal estrogen levels tamoxifen acts as an estrogen-antagonist, competing with 17β-estradiol for the estrogen receptor and causing bone loss [58]. Tamoxifen treatment has led to a statistically significant, 32% reduction in osteoporotic fractures, compared to placebo [21].

13.2.2.2 AIs and Bone

AIs are a class of compounds that block the bioconversion of estradiol from androgens, resulting in very low estradiol levels. AIs can be divided into two groups; competitive or non-steroidal AIs and inactivators or steroidal inhibitors. The non-steroidal AIs, letrozole and anastrozole, are imidazole-based compounds that reversibly bind to the active moiety of the cytochrome P450 enzyme aromatase and block estradiol formation. The steroidal inhibitors, fomestane and exemastane, have an androgen-like structure and compete with the aromatase substrate, androstenedione. They bind covalently and irreversibly to the active site of the enzyme, causing loss of enzymatic activity. This induces an increase in estradiol synthesis. Exemestane also has weak androgenic activity which is attributed to one of its metabolites.

Currently three third-generation AIs are approved by the Food and Drug Administration (USA) for treatment of BCa. All are fairly potent and cause 98% suppression of aromatase activity. Bioconversion of estradiol is the major pathway for estradiol synthesis in postmenopausal women. Hence, all AIs cause a significant lowering of estradiol levels in postmenopausal women. Because estrogen helps maintain bone mass, its deficiency – whether the result of natural or surgical menopause – is a major cause of bone loss, causing women to lose as much as 20% of their bone mass in the first 5–7 yrs after menopause [1,23]. This loss in bone is the basis for an exponential increase in fracture risk after 55 yrs of age [60,77]. In a prospective epidemiological study [15], women with serum estradiol concentrations of less than 5 pg/ml were found to have a higher risk for hip and vertebral fractures than women with serum estradiol concentrations over 10 pg/ml. Because the loss of bone mass at the hip is eight times greater in women with lower estrogen levels, even moderate increases in serum estrogen levels may reduce loss of bone mass and fractures. Therefore the adverse effects of low estrogen levels and increased risks for osteopenia and osteoporosis are a major concern in the treatment with AIs.

13.3 Efficacy of AIs

13.3.1 Studies of Healthy Women on AIs – Effects on Bone Markers

The aromatase gene (CYP-19) is expressed in ovarian as well as non-ovarian tissue, including fat, muscles, skin, liver, and bone, and is a major site of action for the AIs. AIs affect bone metabolism by systemic estrogen suppression and by inhibiting local estrogen production in the bone microenvironment, which results in bone loss.

A few reports have described the effects of AIs on bone in healthy postmenopausal women. Goss et al. [27] performed a randomized, single-blind, placebo-controlled exploratory study to evaluate how low plasma estrogen levels, induced by AIs, affected markers of bone turnover in 80 healthy postmenopausal women during 24 weeks of outpatient treatment, using exemestane, letrozole, and anastrozole. All three inhibitors caused plasma estrogen levels to decrease and bone-resorption markers to increase to the same degree. Exemestane, however also increased the serum levels of the bone-formation marker, P1NP at week 24. This effect was attributed to the androgenic structure of exemestane.

Heshmati et al. [32] compared bone-turnover markers in 42 normal women randomly assigned to receive letrozole or placebo for 6 months. Letrozole treatment reduced serum estrone and estradiol to near undetectable levels ($p < 0.0001$), and caused a significant increase in bone-resorption markers, including urine 24-h pyridinoline (PYD) and 24-h urine deoxypyridinoline (DPD).There was also a significant decrease in serum parathyroid hormone (PTH) levels. Bone-formation markers did not change. Therefore lowering serum estrogen in postmenopausal women with AIs causes increased bone resorption. At the same time the estradiol synthesized by aromatase helps maintain the balance between bone resorption and formation.

Two large, ongoing randomized trials are currently evaluating AIs for the prevention of BCa in healthy postmenopausal women. The Second International Breast Cancer Intervention Study (IBIS-II) is studying the effect of anastrozole in postmenopausal women at increased risk for BCa [16]. The National Cancer Institute of Canada Clinical Trials Group MAP3 study (ExCeL) is evaluating whether exemestane reduces the incidence of invasive BCa in postmenopausal women who are at an increased risk for developing BCa [59].

13.3.2 Data on Premenopausal Women

In premenopausal women with BCa, ovarian ablation therapy with chemotherapy has led to a high rate of bone loss [66]. GnRH analogs have been tried alone or in combination with tamoxifen. Treatment with goserelin, a GnRH analog, has led to a much greater decline in bone density than obtained in adjuvant chemotherapy with cyclophosphamide, methotrexate, or 5-fluorouracil (CMF). In goserelin-treated patients, BMD at the lumbar spine decreased by 10.5%, as compared with 6.5% in CMF-treated patients ($p < 0.001$). The CMF patients remained estrogen-deficient and had ongoing bone loss, whereas patients treated with goserelin had recovered their bone density within one year after the therapy ended [22].

In premenopausal women, AIs, by suppressing peripheral aromatase, cause a reduced feedback to the hypothalamus and an increase in ovarian stimulation [68]. They therefore should be prescribed when the ovaries are removed surgically, by radiation, or with the aid of GnRH analogs. AIs in premenopausal BCa are being evaluated in two large International Breast Cancer Study Group (IBCSG) trials, Suppression of Ovarian Function Trial (SOFT), and the Tamoxifen and Exemestane Trial (TEXT). In SOFT, tamoxifen alone (with or without prior chemotherapy) is compared to tamoxifen and ovarian suppression and to exemestane and ovarian suppression. In TEXT premenopausal patients are randomly assigned to receive the LHRH analog triptorelin with tamoxifen or with exemestane (with or without adjuvant chemotherapy).

13.4 AIs as First-Line Treatment for BCa – Effect on Bone Loss and Fractures

13.4.1 AIs Compared to Tamoxifen

13.4.1.1 Anastrozole

Anastrozole, the first of the third generation of AIs, was compared with tamoxifen for its effects on disease-free survival in 9,366 postmenopausal women with early-stage BCa [8]. Anastrozole significantly improved disease-free survival and significantly lowered cancer in the contra-lateral breast, compared to tamoxifen or combination therapy. Women who received anastrozole had a lower incidence of venous thromboembolic events, pulmonary embolism, ischemic cerebral vascular events, and endometrial cancer than those on tamoxifen. Anastrozole however induced a significantly higher frequency of musculoskeletal symptoms and fractures, 11% versus 7.7% with tamoxifen [3]. Vertebral fractures also increased, but not hip fractures [8].

Changes in BMD and bone-turnover markers with anastrozole were assessed in the bone sub-protocol of ATAC [19]. Anastrozole was associated with statistically significant losses of

BMD at the lumbar spine and hip, both at year 1. Tamoxifen treatment, on the other hand, led to significant gains at the lumbar spine and hip in both years 1 and 2. The decline in BMD slowed after the third year of the study. After 1 year, anastrozole was associated with an increase in bone remodeling as measured by the increase in markers of bone turnover. Tamoxifen caused a decrease in bone remodeling and a significant decrease in bone turnover.

13.4.1.2 Letrozole

Letrozole is currently being evaluated in the Breast International Group (BIG 1-98 5-year study of letrozole, letrozole followed by tamoxifen, tamoxifen, and tamoxifen followed by letrozole). Of 8,010 women who have been enrolled, 4,003 have been assigned to the letrozole group and 4,007 to the tamoxifen group [75]. Clinical fractures at a median follow-up period of 25.8 months were significantly more frequent with letrozole. Other results are not yet available.

13.4.1.3 Exemestane

The Tamoxifen Exemestane Adjuvant Multicenter trial [38] is a phase III, randomized, parallel-group, multicenter trial designed to compare the disease-free survival after 5 years of adjuvant exemestane versus 2.5 years of tamoxifen followed by 2.5 years of exemestane administered to postmenopausal women with early BCa. Data on clinical fractures are as yet unavailable but a bone substudy evaluating BMD has been completed [37]. The number of women with established osteoporosis at baseline was greater in the exemestane than the tamoxifen group. After 12 months of treatment, patients treated with exemestane showed significantly more bone loss at both the spine and hip than patients treated with tamoxifen.

13.4.2 AIs After 2–3 Yrs of Tamoxifen Versus Tamoxifen Alone

Recently third-generation AIs have proven their efficacy and tolerability compared with tamoxifen. The best sequence of administering these medications to hormone-responsive women is under study, as BMD and bone turnover may be influenced by prior tamoxifen treatment.

13.4.2.1 Anastrozole

The Austrian Breast and Colorectal Study Group (ABCSG) 8/Arimidex-Nolvadex (ARNO) 95 study [35] was a prospectively planned, combined analysis of two trials in which sequential treatment with anastrozole administered after 2 years of tamoxifen was compared to tamoxifen alone. After a median follow-up of 28 months, there were significantly more clinical fractures in patients who switched to anastrozole than in those who received only tamoxifen (2% versus 1%, respectively; $P = 0.015$). The clinical fracture rate in the anastrozole group was lower than that in the ATAC trial; this suggests that prior treatment with tamoxifen may have conferred some benefit.

13.4.2.2 Letrozole

The BIG 1-98 study is evaluating sequential treatment with tamoxifen followed by letrozole or letrozole followed by tamoxifen; additional groups receive letrozole or tamoxifen alone [9]. Preliminary results are available at 25.8 months of the study, but the outcome of switching treatment on bone density is not available.

13.4.2.3 Exemestane

Gonneli et al. [26] compared the effects of the steroidal aromatase inactivator exemestane on bone-turnover markers and on BMD in 70 postmenopausal women (62.0 ± 8.9 years) with completely resected BCa who were disease-free following 2–3 years on tamoxifen. The subjects were randomly assigned to continue tamoxifen or switch to exemestane. In the latter bone density at the lumbar spine and the femoral neck declined progressively; this was not true in the tamoxifen group. The bone-turnover markers did not increase significantly in either group.

The Intergroup Exemestane Study compared the effects of tamoxifen administered for 5 years with tamoxifen administered for 2–3 years, followed by exemestane for a total of 5 years in 4,724 postmenopausal women [14]. The incidence of fractures was greater, but not significant in the exemestane group. The incidence of osteoporosis increased slightly with exemestane. In 206 non-osteoporotic patients from 17 centers, BMD at the lumbar spine and the total hip decreased rapidly from baseline after switching from tamoxifen to exemestane. After

6 months the decline in BMD slowed progressively. The total number of fractures over the 24-month period was low and no conclusions were reached on the relationship of fractures to changes in BMD or bone-markers.

13.4.3 Als Versus Placebo After Tamoxifen for 5 Yrs

13.4.3.1 Letrozole

The National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) study (MA.17) studied letrozole therapy after 5 years of standard adjuvant tamoxifen [28,54]. Women receiving letrozole reported a greater incidence of osteoporosis and clinical fractures; neither increase was statistically significant. In a sub-protocol, designed to evaluate bone turnover and BMD in 226 women, patients receiving letrozole at 24 months had a significantly greater decrease in total hip and lumbar spine. Their urine *N*-telopeptide increased significantly at 12 months and 24 months. No patient went below the threshold for osteoporosis in total hip BMD, whereas at the lumbar spine (L2–L4) more women receiving letrozole became osteoporotic.

These studies show that whether administered to otherwise healthy women or as first-line therapy to treat BCa, Als cause bone turnover to increase and BMD to decrease. This is probably the result of reducing estradiol levels. Als thus increase the risk for developing osteoporosis in a population already at increased risk of osteoporosis because of the decrease in endogenous estradiol brought on by menopause.

13.5 Role of Bisphosphonates in Prostate and Breast Cancer

13.5.1 Prevention and Treatment of Bone Metastases

Bisphosphonates are potent inhibitors of osteoclastic function and the mainstay of bone-directed therapy for bone metastases. They act on bone by inducing osteoclast apoptosis, inhibiting osteoclast maturation and differentiation, and reducing osteoclast activity [18]. Bisphosphonates also act directly on tumor cells, inducing apoptosis, inhibiting matrix metalloproteinase 1, inhibiting angiogenesis, decreasing adhesion of tumor cells within bone, and reducing the vascular endothelial growth factor [12]. Bisphosphonate treatment aims to prevent and delay skeletal-related events (SREs), reduce bone pain, and improve quality of life.

13.5.2 Prostate Cancer

PCa is primarily an osteoblastic bone disorder, but osteoclastic resorption has been shown to be an integral part of skeletal metastases in this cancer [10]. Trials to determine if bisphosphonates prevent or delay the onset of bone metastases are underway. Bisphosphonates prevent bone metastases, but this has not yet been shown in PCa [48]. Patients with established bone metastases from PCa appear to benefit from bisphosphonate treatment (MRC PR05 trial). However, oral clondronate given to men at high risk for bone metastases did not change metastases-free survival for up to 10 years. Patient selection may have been a factor in that trial. Yet trials with pamidronate have resulted in equally disappointing results in men with metastatic PCa [67]. Similarly the administration of 4 mg zoledronic acid in men with hormone-refractory, non-metastatic PCa did not affect development of bone metastases [50]. In contrast, in a trial of 634 men with metastasis and hormone-refractory disease, therapy with zoledronic acid reduced SREs including pathologic fractures. Pain control also was improved [62].

Future trials of bisposphonates in PCa are under way. In one trial, men with high-risk disease will receive zoledronic acid [48]. In another, men on long-term ADT for newly diagnosed advanced non-metastatic or metastatic disease will receive hormonal therapy with or without zoledronic acid, docetaxel or celecoxib, or a combination of these [STAMPEOE trial].

Another method of targeting the osteoclast is with an antibody against receptor activator of NF-kappaB ligand or RANK-L, an important ligand in the differentiation and activation of osteoclasts. Denosumab is a monoclonal antibody that targets osteoclasts and inhibits the cell signaling pathway involving maturation

of osteoclasts. Subcutaneous denosumab has been shown to suppress bone-resorption markers [55]. This drug is now in trial in men with hormone-resistant PCa and bone metastases to evaluate whether disease progression can be slowed by denosumab. It is also being evaluated for metastasis-free survival in metastasis-free castrate men who are at high risk of bone metastases. In a third trial, effects of denosumab on BMD and fracture rate are being evaluated in 1,400 men on current ADT.

13.5.3 Breast Cancer

Bisphosphonates have become standard therapy in BCa that has metastasized to bone, according to the guidelines issues by the American Society of Clinical Oncology (ASCO) [33] and the Cancer Care Ontario guidelines. Benefits of the bisphosphonates have become evident from a number of trials and meta-analyses [56]. Pamidronate, ibandronate, zoledronic acid, and clodronate all have been shown to reduce the number of SREs and to increase the length of time before BCa metastasizes to bone [4,34,40,41] The ASCO guidelines recommend that bisphosphonate treatment continues until the patient's general performance status declines substantially.

As with PCa, anti-RANKL therapy with denusomab also is being evaluated in women with metastatic BCa. Lipton et al. [44] compared denusomab with standard intravenous bisphosphonate administration in women with BCa and bone metastasis and found that subcutaneous denosumab, like the intravenous bisphosphonates, suppresses bone turnover and reduces SRE risk.

13.6 Prevention of Bone Loss in Prostate and BCa

13.6.1 Prostate Cancer

Men, like women, experience bone loss and osteoporosis as they age, although to a much lesser extent. Factors that account for the difference are the higher peak bone mass in men [29], their larger bone structure and greater muscle mass, and the absence of accelerated bone loss that women experience around the menopause. Fractures experienced by older men in many instances are due to factors other than age, e.g., hypogonadism, glucocorticoid or GnRH-agonist use, excess alcohol consumption, and smoking [53]. In addition, as in women, men may have vitamin D deficiency, a poor calcium intake, and not engage in weight-bearing exercise. After 50 years of age, 20% of Caucasian men will experience a fracture. Approximately 30% of all hip fractures in the US occur in older men, accounting for 25% of the costs of fracture care [29]. Men with hip fractures have higher 1-year mortality (37.5%) than older women with hip fractures (28%); thus, hip fractures in men constitute a significant marker of frailty.

13.6.2 Prevention of Bone Loss with Bisphosphonates

Men on ADT are at higher risk of bone loss and fractures than otherwise healthy community-dwelling men [43,65,69]. Intravenous pamidronate [72] and zoledronic acid [49] administered to men with metastatic and non-metastatic PCa have improved and increased BMD. A single annual 4 mg dose of zoledronic acid increased BMD in the spine and hip [49].

Greenspan et al. [29] sought to determine whether oral alendronate (70 mg/w) once weekly could prevent bone loss and reduce bone turnover in 112 men receiving ADT in a double-blind, randomized, placebo-controlled trial. All patients received in addition calcium and vitamin D supplementation. After one year, men treated with alendronate had significant gains in BMD, 3.7% at the spine and 1.6% at the hip, whereas men in the placebo group had losses of 1.4% at the spine and 0.7% at the hip.

In our own work [73], a double-blind, placebo-controlled, randomized trial of oral risedronate (35 mg/week) versus placebo in older men receiving GnRH-agonist therapy for locally advanced PCa and who also received daily doses of calcium and vitamin D, we showed that after 6 months, neither femoral neck nor total hip BMD decreased in the risedronate group, whereas there were BMD losses in the placebo group. Spine BMD of the risedronate

group increased significantly by 2.3%, but the placebo group did not change. Thus, GnRH-agonist therapy produces rapid bone loss within 6 months that can be prevented by risedronate treatment.

13.6.3 Use of Estrogen to Prevent Bone Loss in PCa

Medical castration with estrogen, specifically DES, has been the treatment for metastatic PCa. In men who have undergone bilateral orchidectomy, hip BMD decreases by approximately 10% per year, whereas men given oral and intramuscular estrogen for medical castration lose only about 1% hip bone BMD [20]. With transdermal estradiol patches to achieve castration, 20 men with advanced or metastatic PCa showed increases in BMD at the spine and hip after 1 year of treatment [52]. Bone turnover decreases when estrogen is administered intramuscularly to men treated with orchidectomy [9] or DES [64], or to men on GnRH-agonist therapy who are receiving 1 mg/day of oral micronized estradiol [74].

A recent cross-sectional study from Japan [78] showed that combined androgen blockade with GnRH-agonists plus an androgen receptor blocker (CAB) or estramustine, a chemotherapeutic agent with estrogen-like properties, maintains bone turnover and BMD compared with a group receiving androgen deprivation alone. Androgen receptor blockers increase serum concentrations of both testosterone and estradiol. Estradiol is therefore available to tissues [71]. In a 1-year prospective study, men receiving the androgen receptor bicalutamide alone showed increased BMD, less fat accumulation, and fewer bothersome side effects than treatment with a GnRH-agonist [71]. No long-term trials have been performed to determine whether estrogen therapy can improve BMD *and* decrease fractures in men with PCa.

13.6.4 Prevention and Treatment of Bone Loss in Breast Cancer

Association of the newer endocrine therapies for BCa with increased bone turnover and reduction in BMD mandates increased vigilance for monitoring BMD and making sure there are no secondary factors that increase the risk for bone loss. Patients should be educated about the need for adequate calcium and vitamin D intake and a healthy, active lifestyle. Nevertheless, BMD may decline in some patients and may require treatment, as with bisphosphonates.

Animal studies by Gasser et al. [24] provide support for the use of zoledronic acid to prevent or slow bone loss in women with BCa who are on AI therapy. Gasser and colleagues [24] found that daily treatment with the AI letrozole, administered orally, induced significant bone loss and cortical thinning, but that zoledronic acid prevented bone thinning and loss in rats.

Yonehara et al. [79] compared 17 postmenopausal BCa patients receiving anastrozole (1 mg/d) with 10 patients who in addition received 2.5 mg daily of sodium risedronate for 6 months. Whereas in the women who received only anastrozole, BMD and associated T- and Z-scores decreased significantly, in women who in addition received the bisphosphonate, the BMD and associated T- and Z-scores increased significantly from baseline values.

Confavreux et al. [13] analyzed bone loss and bone turnover in postmenopausal women with osteoporosis treated with anastrozole in comparison with osteoporotic women treated simultaneously with anastrozole and risedronate. In the latter, bone loss was prevented at the hip, and bone density increased significantly at the spine. It is thus apparent that risedronate prevents anastrozole-induced bone loss.

The Austrian Breast and Colorectal Cancer Study Group [25] (ABCSG-12 trial) evaluated the role of zoledronic acid in preventing bone loss associated with adjuvant endocrine therapy in premenopausal patients in a randomized, open-label, phase III, four-arm trial comparing tamoxifen (20 mg/d orally) and goserelin (3.6 mg every 28 days subcutaneously) ± zoledronic acid (4 mg intravenously every 6 months) versus anastrozole (1 mg/d orally) and goserelin ± zoledronic acid for 3 years in premenopausal women with hormone-responsive BCa. Endocrine treatment without zoledronic acid caused a significant overall bone loss after 3 years of treatment. The bone loss was significantly more severe in

patients receiving anastrozole/goserelin than in patients receiving tamoxifen/goserelin. BMD remained stable in zoledronic acid-treated patients. In other words, bone loss was effectively inhibited by zoledronic acid given every six months.

Brufsky et al. [6] evaluated whether addition of zoledronic acid to adjuvant letrozole therapy protects against bone loss in postmenopausal women. A total of 602 postmenopausal women with early-stage BCa and osteopenia (T scores ≥ -2.0), who were starting 5 years of oral letrozole, were randomly assigned to receive up-front intravenous zoledronic acid every 6 months versus delayed zoledronic acid (to start only if T scores decreased below –2.0 or if a nontraumatic clinical fracture occurred). At the end of 1 year, BMD in hip and lumbar spine was statistically higher in the up-front zoledronic acid group. Also in the up-front group, mean serum N-telopeptide and bone-specific alkaline phosphatase concentrations decreased significantly by 15.1% and 8.8%, respectively, at 12 months, whereas those markers increased significantly in the delayed group. These findings indicate that up-front zoledronic acid therapy prevents bone loss in the lumbar spine in postmenopausal women who are receiving adjuvant letrozole for early-stage BCa. Similar results were reported by Bundred et al. [7] who found that immediate zoledronic acid therapy for 12 months prevented bone loss in postmenopausal women who were receiving adjuvant letrozole.

13.7 Osteoporosis Prevention and Treatment in Breast and Prostate Cancer Patients

13.7.1 Clinical Monitoring

It is apparent from the many reports cited in this chapter that women with BCa and men with PCa who receive hormonal therapy should have their bone health evaluated, preferably prior to treatment (see Fig. 13.1). This is particularly important as people age, inasmuch as osteoporosis and fracture risks increase with age. Also, more women are being started on AIs as first-line agents for treatment of BCa compared to tamoxifen due to the improved disease-free survival with these agents. The effect of age, plus the decline in BMD due to AIs and ADT, clearly warrant screening for osteoporosis in all women

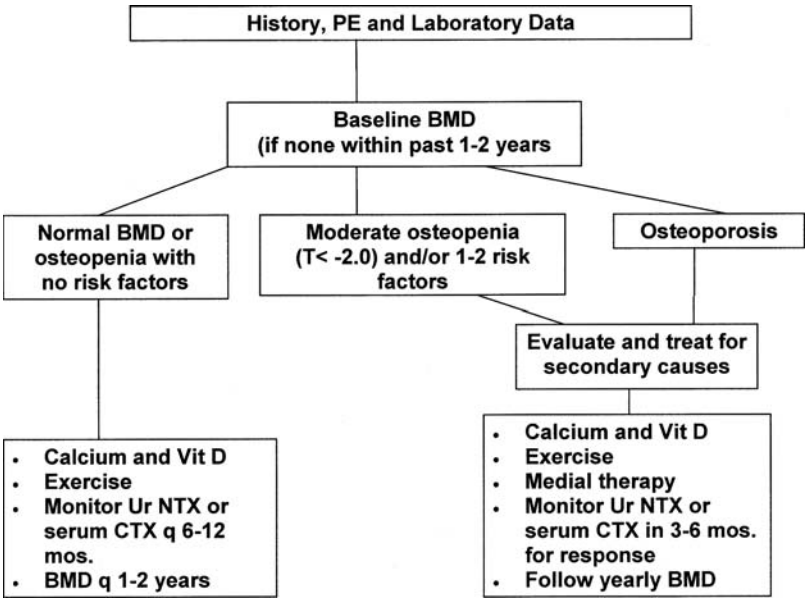

Figure 13.1. Algorithm for evaluation and treatment of hormonal therapy used for prostate and breast cancer.

Table 13.1. Lifestyle interventions

Discontinue smoking
Avoid excess alcohol (> 2 oz per day)
Calcium 1200 mg/day in divided doses
Take carbonate with food, citrate if on H2 blockers or proton pump inhibitors.
Vitamin D_3 (cholecalciferol) 800–1200 IU/day
Exercise
Weight-bearing exercise 30 min 3 times per week
Resistance training

Table 13.2. Laboratory evaluation to r/o secondary causes of osteoporosis

Complete blood count, Serum protein electrophresis
Serum-ionized calcium
TSH
25-hydroxyvitamin D
Serum-ionized calcium, phosphorus, creatinine
Serum alkaline phosphatase, bone-specific alkaline phosphatase, and urine N-telopeptide collagen crosslinks (NTX).

with BCa and all men with PCa. If osteoporosis is already evident, it is important to evaluate for secondary causes (e.g., glucocorticoid therapy, hyperparathyroidism, vitamin D deficiency); see Table 13.1. Calcium and vitamin D intake should be assessed and, if deficient, prescribed at doses listed in Table 13.2. 25-hydroxyvitamin D plasma levels should be above 30–35 ng/ml [17].

Because morbidity and mortality due to osteoporotic hip fractures are significant in both women and men, moderate osteopenia in patients with one or more risk factors or existing osteoporosis should be treated with oral or intravenous bisphosphonates. In the absence of osteoporosis or risk factors, we recommend that urinary NTx or serum CTX be followed at 3–6 month intervals, on the alert for significant increases (of 30–50%). BMD should be evaluated within 6–12 months following initiation of hormonal therapies. We also recommend initiation of anti-resorptive treatment, if turnover markers remain elevated and/ or if BMD declines significantly within 6–12 months of initiation of AI or GnRH-agonist therapy.

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14.

Therapeutic Approaches to Metastatic Bone Cancer II: Targeted and Non-targeted Systemic Agents

Stephen S. Grubbs and Charles J. Schneider

14.1 Introduction

Skeletal metastases occur most commonly in the advanced stages of prostate, breast, and lung cancer, but also can occur in thyroid cancer and renal cell carcinoma (RCC) [22]. The skeleton is the third most common organ affected by metastases from these solid tumors [17]. In general, bone metastases disrupt normal bone remodeling to cause either osteolytic or osteoblastic bone lesions. Metastatic breast cancer and renal cell cancer are often associated with osteolytic bone lesions. Prostate cancer is associated primarily with osteoblastic lesions [8]. Bone metastases cause significant morbidity, including pain, hypercalcemia, pathologic fractures, and spinal cord or nerve root compression. Bone metastases can occasionally lead to life-threatening complications, and most commonly do so for patients with bone metastases from breast and lung cancer [17].

The ability of a tumor to metastasize to bone may be due to the inherent properties of a subset of tumor cells known as metastatic cancer stem cells (mCSCs). The clonal selection model of metastasis hypothesizes that certain genetic mutations acquired by tumor cells in the process of tumorigenesis provide a selective advantage to metastasize to bone [22]. This subset of tumor cells with the ability to metastasize to and thrive in the bone marrow microenvironment constitutes potential targets for therapeutic intervention. For instance, mCSCs have unique surface phenotypes that may facilitate the use of targeted agents directed to the surface antigens. Other therapeutic avenues to explore include encouragement of mCSC differentiation, disruption of the self-renewing abilities of mCSCs by inhibition of pathways that mediate abnormal apoptotic resistance such as NF-κB, and targeting unique niche requirements of mCSCs within the bone marrow stroma or vascular endothelium [19].

The bone microenvironment contains growth factors that promote the engraftment and proliferation of mCSCs. These cancer cells, in turn, produce factors that help sustain growth within bone. Osteolytic factors produced by bone metastases include parathyroid hormone-related peptide (PTHrP), interleukin-6, interleukin-11, and transforming growth factor β (TGF-β) [8]. Bisphosphonates, such as zoledronic acid, may target the unique niche requirements of mCSCs within the bone marrow stroma. Alteration of the bone marrow microenvironment by bisphosphonates may make it difficult for mCSCs to thrive; this can explain the cytotoxic activity of agents such as zoledronic

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acid. For instance, Facchini et al. [17] treated 60 patients with bone metastases from lung or breast cancer with chemotherapy and zoledronic acid, administered in three or more cycles every 3–4 weeks. The bone response was related inversely to chemotherapy and to reduced skeletal-related events (SREs) resulting from zoledronic acid therapy. The authors suggested that cytotoxic synergy between bisphosphonates and chemotherapy was significantly enhanced by the effect of zoledronic acid on the bone marrow microenvironment [17].

Bone pain from osseous metastases occurs because of periosteal invasion by tumor cells, an increase in intraosseus pressure in the bone marrow cavity, and elaboration by the bone marrow stromal cells and the metastatic cancer cells of biochemical mediators and cytokines. Examples of these are prostaglandins, bradykinin, osteoclast-activating bone factor, interleukin-1, and tumor necrosis factor-α (TNF-α) [33]. Proteolytic enzymes and cell surface proteins also have been implicated in the establishment and success of bone metastases and include the matrix metalloproteinases, lysosomal cysteine proteinases, and plasminogen activators. These proteins constitute targets for therapeutic intervention, but clinical results with several matrix metalloproteinase inhibitors including batimastat, marimastat, and prinomastat have been disappointing [43].

Chemotherapy palliates pain in approximately 50% of patients with bone metastases [15]. New targeted agents have been used in conjunction with chemotherapy, but also singly without chemotherapy in some malignancies. This chapter will focus on the use of targeted agents and chemotherapy for treating patients with bone metastases from RCC, thyroid carcinoma, prostate cancer, breast cancer, and lung cancer.

14.2 Renal Cell Carcinoma

RCC frequently metastasizes to bone and, in general, is highly resistant to radiation treatment and chemotherapy [35]. RCC cells overexpress the multidrug resistance protein; this explains, in part, the low response rate to chemotherapy. More importantly, most clear cell RCCs have mutations that result in biallelic loss of the von Hippel–Lindau (VHL) tumor suppressor gene. This gene plays a key role in stimulating angiogenesis by the vascular endothelial growth factor (VEGF), which explains why this tumor is highly vascularized. The VHL pathway and resulting downstream products may be targets for therapeutic agents employed in the treatment of RCC [41].

Traditionally, patients with RCC have been treated with immunotherapy rather than chemotherapy, because RCC cells express the multidrug resistance gene. Newer targeted therapies largely have replaced immunotherapy. Immune stimulation using alpha-interferon or interleukin-2 can induce tumor regression in some patients. Other forms of immunotherapy have shown some promise in the treatment of metastatic RCC. Monocyte-derived tumor-antigen-sensitized dendritic cells are being investigated, not only in patients with RCC, but also in patients with bone metastases from prostate cancer. Monoclonal antibodies directed to RCC antigens, such as G250 (WX G250), are being investigated, as are monoclonal antibodies directed against IL-6. Finally, monoclonal antibodies directed against TNF-α, such as infliximab, show some promise in treating patients with metastatic RCC [41].

An important downstream product of VHL, which is a potential target for therapeutic agents, is hypoxia-inducible factor (HIF). The VHL tumor-suppressor gene product is known as pVHL, which under normoxic circumstances binds the HIF-α to form a complex. In conditions of hypoxia, or due to a defective/mutated pVHL function, the interaction is dysfunctional, resulting in accumulation of HIF-α, which in turn results in the transcription of several hypoxia-inducible genes. Some of these genes produce tumor-active growth factors, such as VEGF and platelet-derived growth factor (PDGF). The growth factors play an important role in angiogenesis, cell proliferation, and cell survival [41, 35]. Some novel agents that disrupt angiogenesis by inhibiting HIF downstream products include bevacizumab, PTK787, SU11248, gefitinib, cetuximab, erlotinib, Bay43-9006, and ISIS-5132 [35].

Anti-angiogenesis drugs have shown promise in treating RCC, largely due to the HIF-induced increase in angiogenesis described above. Thalidomide inhibits angiogenesis by down-regulating HIF-induced production of VEGF. Neovastat (AE-941), obtained from shark cartilage, inhibits several HIF-induced VEGF-dependent processes. Several small molecules interact with the HIF-induced VEGF pathway. Sunitinib (sutent) is a multitarget inhibitor that interacts with VEGF, PDGFR, KIT, and FLT3R tyrosine kinases. PTK787/ZK2222584 is a selective inhibitor of VEGFR-1, VGEFR-2, and PDGFR-beta tyrosine kinases [41].

Bevacizumab is a humanized monoclonal antibody that binds all subtypes of HIF-induced VEGF and has substantial activity in RCC [41]. In a study involving 116 patients, bevacizumab (10 mg/kg) significantly slowed disease progression. The probability of being progression-free (PF) at 8 months was 14% for the low- (3 mg/kg) and high-dose (10 mg/kg) groups combined, compared with a probability of 5% for the placebo group. There was no appreciable difference in overall survival, because patients on placebo were unblinded at the time of disease progression and received bevacizumab instead [44].

The Raf kinase pathway is complex and constitutes another therapeutic target for patients with metastatic RCC. RAF proteins play an essential role in regulating normal cell proliferation and apoptosis. Many agents interfere with this pathway by blocking expression of the protein, or by interfering with the kinase activity of the activated protein. A specific inhibitor of Raf-1 kinase is sorafenib (Nexavar), an oral inhibitor of Raf and also of other targets such as the receptor kinases of VEGFR-2, VEGFR-3, FLT-3, PDGFR, and c-KIT [7]. In a phase III randomized placebo-controlled trial of 905 patients, who were cytokine-refractory, sorafenib yielded a PF survival in 2% of patients, and resulted in stable disease in 78% of patients. Tumor shrinkage or stabilization was observed in 74% of patients taking sorafenib, compared with 20% of patients receiving placebo. Median PF survival was 24 weeks in the sorafenib group, compared with 12 weeks in the placebo group. Preliminary results also showed improved overall survival in all patient subgroups receiving sorafenib [16].

Sunitinib is another Raf kinase inhibitor. Two phase II studies using sunitinib in patients with RCC who failed first-line treatment with cytokines showed significant results. In the first study of 64 patients, 40% achieved a partial response, with 33% disease-stable. The clinical benefit ratio, defined as an objective response plus disease stability, was greater than 70%. Of the 25 patients achieving a PR, the median duration of response was 10+ months. Median time to progression was 8.3 months, and median overall survival was 16 months. In the second trial, preliminary results reported an objective response in 29% of 83 patients [28].

The mammalian target of rapamycin (mTOR) regulates signal transduction pathways that involve cell cycle progression, cell proliferation, cell survival, cell mobility, and angiogenesis. Mutations in mTOR are prominent in RCC cells. CCI-779 (temsirolimus) is a specific inhibitor of mTOR, which has activity against metastatic RCC. In a randomized double-blind phase II study, an objective response was observed in 7% of patients, and minor responses were observed in 26% of patients [3]. Temsirolimus has been approved by the Food and Drug Agency (USA) for use in patients with refractory metastatic RCC.

14.3 Thyroid Cancer

14.3.1 Well-Differentiated Carcinoma of the Thyroid

Targeted therapy of bone metastases due to a well-differentiated thyroid cancer exploits the avidity for iodide by thyroid cells to deliver radiation from ^{131}I directly to the bone metastases, thus destroying them [40].

14.3.2 Medullary Thyroid Carcinoma

Thyroid carcinoma cells do not bind iodine and therefore cannot be treated with ^{131}I. No effective systemic treatment is known

to treat distant metastases from MTC [12]. Chemotherapeutic agents that have been evaluated include 5-fluorouracil, doxorubicin, and etoposide (FDE); doxorubicin and streptozotocin; and 5-fluorouracil with dacarbazine (DTIC) [26].

Hormone therapy of medullary thyroid carcinoma (MTC) with octroetide, a somatostatin analog that inhibits neuroendocrine tumor cell growth by inhibiting the release of growth-promoting hormones, by inhibiting angiogenesis and by modulating immunologic activity, has been conducted. Somatostatin analogs, by binding to somatostatin receptors, have a direct cytotoxic effect on tumor cells. Because MTC cells have surface receptors for somatostatin, somatostatin analogs have been evaluated, but while symptoms have improved in some patients because hormone release by tumor cells is reduced, octreotide does not reduce tumor mass or improve survival [12].

Because C cells secrete carcinoembryonic antigen (CEA), anti-CEA antibodies have been utilized therapeutically. Sharkey et al. [34] showed that a humanized anti-CEA antibody (labetuzumab) inhibited MTC tumor growth in vivo and sensitized tumor cells to responding to DTIC treatment. These promising laboratory observations notwithstanding, a phase-1 trial utilizing radiolabeled labetuzumab in conjunction with high-dose doxorubicin and peripheral blood stem cell rescue showed limited benefit in patients with advanced MTC [18].

Because mutations in the RET proto-oncogene are a component of hereditary MTC, the RET product could be a target for treating patients with MTC. RET encodes a transmembrane tyrosine kinase receptor. Ligands for RET include a glial-derived neurotrophic factor known as GDN, along with three other members of this family: neurturin, artemin, and persepherin. The RET tyrosine kinase receptor binds with two copies of its co-receptors, activating multiple downstream pathways including ERK, P13K/AKT, p38 MAPK, and JNK. Receptor blockade or inhibition induces apoptosis in MTC cells. Small-molecule tyrosine kinase inhibitors under study for MTC include ZD6474 (Zactima), sorafenib (Nexavar), sunitinib (Sutent), and imatinib mesylate (Gleevac) [4].

14.3.3 Anaplastic Thyroid Carcinoma

Anaplastic thyroid carcinoma (ATC) has a rapidly fatal course, with median survival after diagnosis of 4–12 months. Systemic metastases occur in 57–75% of patients, with lung being the most common site (80%), followed by bone (6–15%) and brain (5–13%). Chemotherapy agents that have significant activity against ATC cell lines and have clinical utility include doxorubicin, paclitaxel, vinorelbine, gemcitabine, bleomycin, cyclophosphamide, 5-fluorouracil, cisplatin, and mitoxantrone [42].

The response rate of doxorubicin therapy is no greater than approximately 20%. Combination therapy using doxorubicin with cisplatin or bleomycin gives no better clinical response than doxorubicin therapy alone. Addition of paclitaxel improved the response somewhat, but had no effect on the eventual fatal outcome [30]. In a phase II trial of 19 patients with persistent local or metastatic ATC, infusion of taxol (120–225 mg/m^2 over 96 h) produced a response rate of 57%, with one response complete. Responses, however, were of short duration [1].

The epidermal growth factor receptor (EGFR) may be a promising target for treating ATCs, because approximately 40% of ATC cases overexpress this receptor [14]. Because ATC cells consistently overexpress EGFR, gefitinib (Iressa) can effectively block activation of EGFR by EGF, leading to inhibition of ATC proliferation and inducing apoptosis. Studies in a subcutaneous nude mouse model showed that gefitinib had significant antitumor activity. It therefore can be a candidate for use in human clinical trials [18].

The ubiquitin-proteosome pathway is a major pathway for the degradation of intracellular proteins. Bortezomib (Velcade) is a proteosome inhibitor that induces apoptosis in medullary and anaplastic thyroid cancer cell lines. The combination of bortezomib with the chemotherapy agent, doxorubicin, appears to be synergistic [25].

14.4 Prostate Cancer

Prostate cancer metastasizes almost exclusively to bone, where bone lesions are primarily osteoblastic, or 'bone-forming.' Although the

mechanisms that induce osteoblastic response are poorly understood, in vitro studies have shown that prostate cancer metastases secrete soluble factors that induce proliferation and differentiation of osteoblast precursors. Soluble factors that induce these cellular reactions include bone morphogenetic proteins (BMPs), insulin-like growth factor-1 (IGF-1), endothelin, and ligands for the low-density lipoprotein receptor-related protein 5 [31].

Cytotoxic agents have not been routinely used in hormone-refractory prostate cancer that has metastasized, because of the widely held belief that prostate cancer does not respond to chemotherapy. Modern treatment strategies for hormone-refractory prostate cancer have now evolved to include cytotoxic agents, inasmuch as chemotherapeutic agents, when used in conjunction with corticosteroids, offer effective palliative treatment for patients with metastatic disease [29].

Docetaxel is the most widely used chemotherapeutic agent for patients with hormone-refractory prostate cancer. The taxane drugs exert their effects by binding to and causing polymerization of the mitotic spindle apparatus microtubules; this results in cell instability and cell cycle arrest. Docetaxel, a semisynthetic analog of paclitaxel, exhibits significantly greater cellular affinity and uptake and slower cellular efflux than paclitaxel. This effectively prolongs the length of time the cancer cell is exposed to the drug. The taxanes also induce apoptosis via phosphorylation and inactivation of the anti-apoptotic protein bcl-2, activation of the jun N-terminal kinase (JNK) pathway, and activation of the caspase signaling pathway [29].

The Tax 327 study compared docetaxel and steroids to mitoxantrone and steroids. The most effective treatment was docetaxel 75 mg/m^2 administered every 3 weeks; it led to statistically significant improvement in overall and median survival. Patients receiving docetaxel plus steroids also enjoyed significant improvements in pain (35% vs 22%), prostate-specific antigen (PSA) response (45% vs 32%), and quality of life, compared to the mitoxantrone plus steroid group [38].

Zoledronic acid, at a concentration found in bone, enhances the anti-tumor activity of docetaxel in the hormone-resistant prostate cancer cell line PC-3, although the osteolytic rather than osteoblastic nature of this cell line may be a factor in the results. Cells were exposed to zoledronic acid in combination with or in sequence to docetaxel. Cell viability, apoptosis, and markers for inhibition of the mevalonate pathway were analyzed 48 h and 72 h after drug treatment. Reduction in cell viability and increases in apoptosis were most pronounced when zoledronic acid was given alone. This suggests that patients with bone metastases due to prostate cancer may experience tumoricidal benefit from zoledronic acid without chemotherapy. This in vitro finding supports the hypothesis that bisphosphonates are cytotoxic even without accompanying chemotherapy. Presumably they alter the bone marrow stroma microenvironment in a way that decreases cell stability and their ability successfully to metastasize to the bone marrow [27].

The endothelin-A (ETa) antagonist atrasentan has shown promise in the treatment of patients with hormone-refractory prostate cancer. It inhibits growth of prostate cancer cells by blocking the ETa/ET-1 transduction pathway, producing a dose-dependent suppression of bone formation markers. Preliminary studies suggest improvement in pain, and a decrease in the markers of bone progression [20].

Finally, the vitamin D metabolite, $1,25(OH)_2D_3$, may play a beneficial role in the treatment of bone metastases in patients with prostate cancer. The direct actions of $1,25(OH)_2D_3$ on the bone-forming unit are complex. $1,25(OH)_2D_3$ can induce bone resorption, a process that is mediated by upregulating the expression of RANKL (osteoclast differentiating and activating factor). In tissue culture and in animal models, $1,25(OH)_2D_3$ and its analogs inhibit proliferation of malignant cells and induce cancer cell differentiation and apoptosis. For this reason, $1,25(OH)_2D_3$ and its analogs are being studied in patients with bone metastases from prostate cancer [31]. For example, Beer et al. [6] found that treatment with $1,25(OH)_2D_3$ and docetaxel of patients who had advanced, androgen-independent prostate cancer was more effective than docetaxel treatment alone in causing a decrease in plasma levels of PSA and other measurable parameters of disease

[6]. ASCENT (K), a large phase III placebo-controlled double-blinded randomized clinical trial, is currently being conducted at 58 medical centers to determine whether $1,25(OH)_2D_3$ enhances the effectiveness of docetaxel and glucocorticoids therapy in patients with advanced prostate cancer [5].

14.5 Breast Cancer

Chemotherapy can provide significant palliation for patients with bone metastases due to breast cancer. Among the most active agents currently in use for these patients are the taxanes, anthracyclines such as doxorubicin and epirubicin, gemcitabine, navelbine, and 5-fluorouracil. Most other agents are currently used in combination with chemotherapy [24].

Paclitaxel has been studied in combination with other agents. A phase III randomized trial [24] compared paclitaxel to paclitaxel plus bevacizumab in 722 patients with metastatic breast cancer. The primary endpoint was PF survival, and the secondary endpoint was overall survival. The combination was significantly better than paclitaxel alone, with PF survival of 11.98 months compared to 5.9 months, and an objective response rate of 36.9% vs 21.2%. The hazard ratio for progression was 0.60, favoring the combined treatment arm. In a small group of patients with metastatic breast cancer, the addition of trastuzumab to taxane chemotherapy increased the response rate to 83.3%, compared with 33.3% in the group that received only trastuzumab [32].

Lapatinib is a tyrosine kinase inhibitor that targets the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor-2 (HER2), both of which are frequently overexpressed in breast cancer. When HER2 is used as first-line monotherapy for advanced breast cancer, objective responses are seen in 28% of patients [18]. In a phase II study the efficacy of lapatanib, the EGFR/human epidermal growth factor receptor type 2 (HER2) tyrosine kinase inhibitor was assessed in patients with both HER-2 positive and HER-2 negative metastatic breast cancer. The majority of these patients had previously been treated with four or more chemotherapy regimens, with 4.3% of the HER-2-positive patients responding. Clinical benefit was observed in 6%, with a PF interval of 6 months. The HER-2-negative cohort did not respond [9]. In a phase III trial of 339 women with HER2-positive advanced breast cancer, capecitabine plus lapatinib was superior to capecitabine alone. The addition of lapatinib prolonged time to progression with a hazard ratio (HR) of 0.57 ($p = <0.001$), and provided a trend toward improved overall survival (HR 0.78, $p = 0.177$) [10].

Osteoclasts mediate bone destruction in breast cancer skeletal metastases. The proteinase cathepsin K is secreted by osteoclasts, degrades bone, but is also expressed by breast cancer cells that metastasize to bone. A cathepsin K inhibitor (CKI), used in a mouse model, caused a 79% decrease in osteolytic lesions [21]. This agent has not been studied in humans.

14.6 Lung Cancer

About 30–41% of patients with non-small cell lung cancer (NSCLC) develop skeletal metastases, and 45–50% develop SREs [39]. Cumulative survival rates after bone metastases from lung cancer are 59.9% at 6 months, 31.6% at 1 year, and 11.3% at 2 years. The mean survival for patients with osseous metastases from lung cancer is 9.7 months. The prognosis is somewhat more favorable in women, in patients with adenocarcinoma, with solitary bone metastases, without metastases to appendicular bone, in patients with no pathologic fractures, with the Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, or in patients who receive systemic chemotherapy or an EGFR inhibitor [36].

In October 2006, the Food and Drug Administration (USA) approved bevacizumab for use in combination with carboplatin and paclitaxel to treat patients with locally advanced, recurrent, or metastatic, nonsquamous NSCLC. FDA approval was based on the results of a randomized, open-label phase III trial that had been conducted by ECOG. In that trial,

777 chemotherapy-naïve patients with Stage III or IV NSCLC received carboplatin and paclitaxel alone or in combination with bevacizumab. The latter had a significantly better and longer (by ~2 months) overall survival, than did the patients who did not receive bevacizumab [11].

Up to 10% of patients with NSCLC responded to EGFR tyrosine kinase inhibitors, such as erlotinib or gefitinib. Response is better in non-smokers, in women, in patients with adenocarcinoma, and in persons of Asian ethnicity. Molecular analysis shows that somatic mutations in the EGFR tyrosine kinase domain are a strong predictor of response. The L858R point mutation and the E746-A750 deletion constitute 90% of the mutations in responding patients [9]. In a phase II trial reported by Tamura et al. [37], patients with advanced NSCLC received gefitinib if they harbored EGFR mutations. Of the 118 patients screened for mutations, 32 patients had the mutations and 28 were enrolled in the study. The overall response rate to gefitinib was 75%, the disease control rate was 96%, the median PF survival was 11.5 months, and the 1-year survival rate was 79%. Median overall survival has not yet been reached.

14.7 Radiopharmaceuticals

Bone-seeking radiopharmaceuticals constitute an effective palliative and therapeutic intervention for patients with bone metastases. Several bone-seeking radiopharmaceuticals are in current use to identify bone scans in patients with osteoblastic, or mixed osteoblastic and osteolytic bone metastases. Of these, 153Samarium lexidronam (153Sm-EDTMP, Quadramet, Cytogen, Samarium) is designed for deposition in bone metastases. In a study of 32 patients with painful bone metastases from hormone-refractory prostate cancer who received this radiopharmacetical, palliation was observed in 23 patients (72%) for 3 months, with minimal hematologic toxicity [13]. It is conceivable that combining 153Sm-EDTMP with bisphosphonates, chemotherapy, and/or radiation may bring about substantial improvement in palliation [2].

Another bone-seeking radiopharmaceutical currently in use is ^{89}Sr (Metastron, Strontium). ^{89}Sr is a pure beta emitter with a maximum energy of 1.49 MeV and a physical half-life of 50.5 days. In 79 patients with bone metastases, 19 of whom had breast cancer, and 61 of whom had prostate cancer, four radiopharmaceuticals were assessed for effect on pain, quality of life, and bone marrow function [23]. Seventy-three percent of patients reported pain relief, 15% were able to discontinue analgesics. There was an overall improvement in Karnofsky PS from 70% to 78% at the 112-week endpoint. Ten patients had grade 1 or 2 thrombocytopenia, which was reversible within 12 weeks after receiving the radiopharmaceutical treatment dose. There were no significant differences between the four radiopharmaceuticals.

14.8 Conclusion

The modern oncologist possesses a variety of systemic therapies for bone metastases in addition to traditional palliative localized radiation therapy. Conventional chemotherapy options are now augmented by an expanding array of targeted molecules. Bisphosphonates and radiopharmaceuticals offer additional palliative options. Curative therapies remain elusive, but the expanding knowledge of cellular transduction pathways, of the interaction with the bone matrix, and of future clinical trials to develop therapeutics offers optimism for eventual curative strategies.

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15.

The Role of Allogeneic Bone Marrow Transplant in Cancer Treatment

Jose Francisco Tomas and Sergio A. Giralt

15.1 Introduction and Historical Perspectives

Based on the observations [10,53] in animal models that the lethal myelosuppression induced by total body irradiation (TBI) could be overcome by the infusion of un-irradiated bone marrow, hematopoietic cell transplantation has evolved from idea to a well-established therapy used in the treatment of thousands of individuals around the world.

Dr E. Donnall Thomas, who earned the Nobel Prize for his pioneering work in the field, reported in 1957 the results of treating two patients suffering from advanced leukemia with supra-lethal radiation, followed by an infusion of marrow from their identical twins [88]. The prompt engraftment of both patients demonstrated the feasibility of the approach. Further attempts at marrow transplants using donors other than identical twins were made over the next decade. The first patient engrafted using allogeneic marrow was reported by Mathe in 1965 [56], but the patient died of complications probably related to graft-versus-host disease (GVHD).

A number of critical laboratory studies, performed during the 1950s and 1960s, were the basis for the first successful allogeneic transplants in humans. These included the discovery by Dausset [25] and van Rood [93] of a number of antigens expressed on human leukocytes (human leukocyte antigens or HLAs). The first therapeutically successful human marrow transplants were performed in three infants with severe combined immunodeficiency who received transplants from their HLA-matched siblings [7,26,38]. These severe immune-deficient patients did not require a preparative regimen to prevent graft rejection to eliminate disease.

The first successful transplants requiring a preparative regimen were reported in 1972 by a Seattle group that used a preparative regimen with high-dose cyclophosphamide and post-transplant methotrexate in patients with aplastic anemia [87].

In 1977 Thomas and colleagues published a follow-up of the first 100 patients transplanted for advanced leukemia. Their report provided convincing evidence that transplantation could cure patients with refractory acute leukemia [86].

In the early days of stem cell transplants (SCTs) it was thought that the curative effect of allogeneic transplant was primarily due to the high doses of chemo-radiotherapy, with the donor bone marrow simply allowing for hematopoietic recovery in an adequate period of time. It is now apparent

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that the true success of transplantation is not only due to dose intensity, but involves a powerful donor immune response against the host malignancy, a phenomenon known as the graft-versus-tumor (GvT) effect [18].

In the late 1990s myeloablation was shown not to be necessary for a full donor hematopoiesis. As a result it is now possible to utilize the anti-tumor activity of the transplant, yet reduce the toxicity due to the high-dose preparative regimen [40,81,83]. This new technique, known as reduced-intensity conditioning (RIC), has made it possible for older and more debilitated patients to benefit from the GvT effects. In this chapter we will summarize the current role of allogeneic stem cell transplantation in the treatment of patients with cancer.

15.2 Principles and Technical Aspects of Stem Cell Transplantation

The primary components of all hematopoietic transplants are schematically represented in Fig. 15.1 and include:

a) Recipient: The patient who has a malignancy, an intrinsic bone marrow disorder, or a genetic disorder.
b) Donor (the provider of cells): When the cells are provided by the patient to himself, it is known as an autologous transplant; when the cells are provided by a donor it is known as an allogeneic transplant. When the donor is an identical twin it is referred to as syngeneic transplant.
c) Preparative regimen: Physical and chemical agents that provide immunosuppression to the patient who is to receive an allogeneic transplant so as to prevent rejection of the donor graft.
d) Stem cell source: Source of the hematopoietic progenitor cells, usually cytokine primed stem cells from blood or bone marrow, although cord blood also can be used.
e) Post-transplant supportive care.

Allogeneic transplantation is usually performed in patients with diseases that primarily affect the patient's bone marrow (i.e., leukemia or bone marrow failure syndromes); autologous transplantation is usually performed in patients with malignancies that spare the bone marrow, such as lymphomas and some solid tumors.

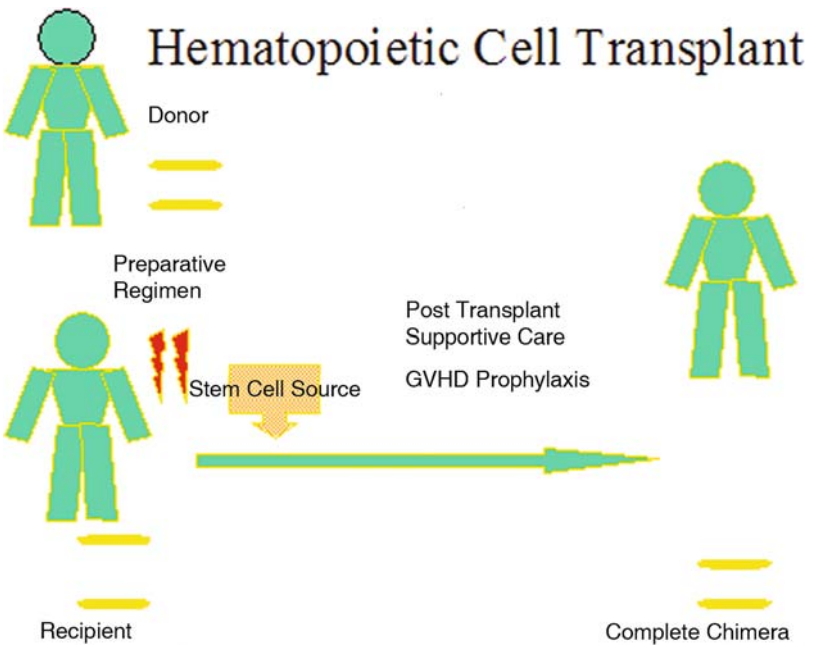

Figure 15.1. Components of allogeneic hematopoietic transplantation.

Transplant conditioning regimens are generally chemotherapy-based or TBI-based protocols. TBI is both immunosuppressive and myeloablative. TBI is associated with a number of short- and long-term complications including secondary malignancies, cataracts, and endocrine dysfunction.

The toxicities of TBI-based regimens led to the development of radiation-free conditioning regimens. Of these, the most commonly used chemotherapy is the combination of busulfan and cyclophosphamide, developed by Santos and coworkers [75] and modified by Tutschka et al. [92]. High plasma levels of busulfan and cyclophosphamide are associated with increased incidence of hepatic veno-occlusive disease (VOD) and other toxicities [57, 92,80]. An intravenous formulation of busulfan is now available that allows dosing once or twice daily with more predictable delivery [3,4].

Historically, hematopoietic stem cells used for transplantation bone marrow cells were obtained from the posterior iliac crests of the donor [86]. Because complications associated with this procedure are very low, it can generally be performed on an outpatient basis. Increasingly, since the early 1990s, peripheral blood-derived stem cells (PBSCs) have been used in preference to bone marrow stem cells, inasmuch as the PBSCs are derived from bone and their use has led to accelerated recovery of hematopoiesis when compared to bone marrow [12,13,51,76].

Umbilical cord blood (UCB) is another rich and easily accessible source of hematopoietic stem cells [74]. Because cord blood cells are less immunocompetent than adults cells, the risk of inducing GVHD may be lower than with adult marrow cells. The small volume of available cord blood (50—150 mL) makes for low stem cell doses in adult patients. Cell dose is an important predictor of engraftment and outcome. UCB has therefore been explored primarily for use in children in whom outcomes are like those in patients who receive stem cells from related or unrelated donors [71,72,74,94]. Improvement in cord blood procurement and storage techniques and new approaches, such as double cord blood transplants or the transient support from CD34 cells from haploidentical family members, have increased the use of cord blood cells in adults [32, 71,72].

15.3 Current Indications for Allogeneic Stem Cell Transplantation in Cancer Patients

15.3.1 Acute Myeloid Leukemia (AML)

With the exception of acute promyelocytic leukemia, allogeneic SCTs offer the highest antileukemic potency if an AML patient is in remission after conventional therapy. Because mortality is increased as a result of the transplant, randomized trials comparing chemotherapy with allogeneic or autologous SCT led to conflicting results [17,20,43,47,68,84,96,99]. In AML patients whose prognosis is poor because of adverse cytogenetics, secondary leukemias, or minimal residual disease (MRD), therapy with allogeneic SCT in CR1 is strongly indicated. The use of RIC has extended this therapy to patients in their sixth and seventh decades of life [11,16,31,40,52,61,62]

Patients beyond first remission do less well with allografting than first remission patients, but allografting is still the best approach to obtain long-term disease control. Patients with chemosensitive disease, long first remissions, and good performance status do reasonably well with allografting [90], but even patients with refractory disease have better outcomes than those treated with conventional chemotherapy. Transplants with stem cells from the best available donor should therefore be considered for patients in remission, whether the first or later, as well as for patients with refractory disease.

Because only 20–30% of patients have a match sibling donor, alternative sources, such as matched unrelated donors, cord blood, or mismatched related transplants should be considered. Aversa et al. [6] used haploidentical SCT in 66 patients with high-risk AML and found event-free survival (EFS) in nearly 60% of those

transplanted in CR, as opposed to 16% of non-transplant patients.

15.3.2 Acute Lymphoblastic Leukemia (ALL)

Pediatric ALL is one of the most successful stories in oncology. What used to be uniformly fatal can now be cured in more than 80% of patients. Moreover, a substantial fraction of relapsing patients can be rescued with chemotherapy, with or without SCT [66]. In contrast to the excellent results achieved in pediatric patients, outcomes for adult ALL patients are disappointing [46]. Currently nearly 90% of adult patients complete CR, but only one-third will be long-term survivors. Factors that predict a low probability of cure include high WBC count at diagnosis (>30,000/μL for B-cell lineage and > 100,000/μL for T-cell lineage), cytogenetics, time to attain CR1, presence of MRD by flow cytometry at day 30, and extramedullary disease. For these patients most of the investigators recommend an allogeneic transplant [45,69,73,85,89]. In the largest trial reported to date [73], 1826 adult ALL patients were given induction therapy and 91% did CR. All patients younger than 50 years old with a matched sibling donor received a myeloablative allogeneic SCT with TBI. A total of 389 patients with a donor were compared with 524 controls. EFS and OS were statistically superior in the donor group: 50% vs. 41% (p: 0.009) and 53% vs. 45% (p: 0.02). The optimal allogeneic effect was observed in the standard-risk rather than the high-risk group, with the groups defined by age and WBC count. Other studies have not supported allografting in adult ALL patients, with the procedure used primarily in patients with high-risk disease [45,85,89].

Even though results for patients in later remissions, early relapse, or primary refractory disease are inferior to those obtained in patients in CR1, an allogeneic SCT improves outcomes when compared to other therapies [34].

15.3.3 Chronic Myeloid Leukemia (CML)

Before the development of the tyrosine kinase inhibitor (TKI), imatinib mesylate, allogeneic SCT was considered the best treatment for CML, with 5-year disease-free survival rates attaining 85% [67]. With the advent of imatinib and other new TKIs allogeneic transplantation is no longer the first option for CML patients [41], but is limited to patients in chronic phase who failed one or, in some instances, two lines of TKIs [42].

In weighing the allogeneic stem cell graft vs. treatment of the leukemia, a non-myeloablative SCT seems an attractive strategy. However, in patients under 50 years of age, a reduced-intensity allo-SCT offers a major advantage to conventional conditioning [24].

Patients with accelerated, blastic, or second chronic phase CML cannot be cured with imatinib and even though clinical results of transplantation are poor for these types of CML, transplantation remains the only potential cure [95].

15.3.4 Myelodysplastic Syndromes (MDSs)

Allogeneic transplantation is the only curative treatment for MDS. Because patients with MDS are older, transplantation has generally been limited to patients with higher risk or advanced disease, in whom the relapse rate is appreciable.

Pre-transplantation induction chemotherapy is as yet controversial [78], even though Yakoub-Agha et al. [98] reported that patients with secondary MDS who achieved remissions with pre-transplant chemotherapy had a substantially better relapse-free survival after SCT than patients who did not achieve a remission [98]. A retrospective analysis indicated that pre-transplant chemotherapy reduced the risk of post-transplant relapse, but failed to be of advantage for post-transplant relapse-free survival. These observations suggest that responses to pre-transplant chemotherapy may select for those patients who fare better after transplantation, even without prior therapy [28].

A report from IBMTR [79] in 452 MDS patients with transplants from HLA-identical siblings showed the best results in younger patients with a low tumor burden (<5% blasts). A conditioning regimen without TBI was best when the donors were not related to the patients [21]. With pharmacokinetic monitoring of oral busulfan, Deeg et al. [28] obtained a

relapse-free survival for 3 years. Survival ranged from 80% for patients with low IPSS to 29% for those with a high IPSS score [28]. Because MDS patients are older and experience comorbidity, developing less-toxic regimens with RIC and non-myeloablative conditioning is of great importance. Inasmuch as relapse is the main cause of failure for this approach [27,52,55,60], it is important to define the optimum conditioning regimen so as to be able to extend this therapy to more patients [30].

15.3.5 Lymphoid Malignancies (Non-Hodgkin's Lymphoma; Hodgkin's Lymphoma, Multiple Myeloma and Chronic Lymphocytic Leukemia)

Autologous SCTs are well established as curative therapy for relapsed Non-Hodgkin's lymphoma (NHL) and Hodgkin's Lymphoma. Clinical results of autologous SCT for primary resistant Hodgkin's disease (HD) or non-chemosensitive relapse patients are poor [14,44,77].

Conventional allografting has led to extremely high rates of non-relapse mortality [36]. The possibility of using non-myeloablative or a RIC regimen in this group of patients seems very promising [1,2,58]. Peggs et al. [63] have shown that long-term disease control is feasible after RIC allografting, particularly in patients with chemosensitive relapses and minimum tumor bulk [63].

Chemosensitive relapse of follicular lymphoma occurred in some of the best reported outcomes for reduced-intensity regimens [49,59]. Patients with follicular lymphoma have many treatment options that provide long-term disease control without the risk of GVHD. Allogeneic transplantation for follicular lymphoma continues to be explored as one therapeutic approach.

Patients with diffuse lymphoma who are chemorefractory do poorly. Whether an autologous transplant followed by a reduced-intensity allograft can change the poor outcome is yet to be determined [19,64,70]. In cases of aggressive NHL, RIC allografting is particularly encouraging for mantle cell lymphoma (MCL). Khouri et al. [50] reported a 82% EFS and 86% overall survival in 18 patients undergoing reduced-intensity regimen with fludarabine/cyclophosphamide. Maris et al. [54] also reported encouraging outcomes for patients with recurrent MCL that had undergone an allogeneic transplant after a low-dose TBI. Overall survival of 65% at 2 years compares favorably with fludarabine-containing regimens and may become future phase III trials [54].

Chronic lymphocytic leukemia (CLL) has been considered a good candidate for RIC allografting [48]. Dreger et al. [29] reported that in 77 patients with CLL the NRM rate was 18% at 1 year and the EFS was 56% at 2 years. These findings should be compared with other non-transplant therapies in patients failing frontline therapy [29]. Sorror et al. [82] reported the results of a low-dose TBI RIC regimen for the treatment of advanced CLL, with a NRM of 22% at 2 years, and a 52% EFS at 2 years. This strategy also warrants further study in CLL patients who relapse after primary therapy.

A diagnosis of multiple myeloma is the most common indication in the US and Europe for the use of autologous SCT. This is so because multiple myeloma is one of the few hematological malignancies in which the impact of dose intensity has been demonstrated and confirmed in large randomized trials [5,22]. The median remission after autologous transplant is around 3 years, and over 90% of patients will have recurrent disease by 10 years post-transplant [9]. A graft-versus-myeloma effect has been demonstrated [91]. However, transplant-related mortality (TRM) in myeloma patients using conventional regimens has been unacceptably high [35].

Data with RIC regimens are encouraging and based on the above high transplant-related mortality. Several studies have demonstrated that TRM was decreased with RIC regimens, but the relapse rate is greater than with standard allografting [23]. The principal factors determining outcome were the state of the disease at transplantation and the presence of chronic GVHD. Two large comparative trials have demonstrated a potential benefit for patients who underwent a RIC allograft as consolidation of an autologous SCT for primary therapy of myeloma [15,37].

15.4 Transplant-Related Complications

Appropriate supportive measures to deal with transplant complications depend largely on what complications the patient may face at a given post-transplant date. The temporal relationship of common infectious and non-infectious complications is depicted in Fig. 15.2. Nausea, dehydration, and gastrointestinal symptoms associated with the conditioning regimen can be managed by standard procedures and with careful monitoring of fluid and electrolyte status [8].

GVHD is the most important limitation to successful transplantation. Clinically acute GVHD involves a maculopapular skin rash, enteritis involving the distal small bowel or colon, and hepatitis. A particular organ or a group of organs may be involved. Acute GVHD generally presents within 100 days after transplantation, but may also present later [33]. The best therapy for GVHD is prophylaxis. The prophylactic use of methotrexate and a calcineurin inhibitor (cyclosporine or tacrolimus) reduces the acute GVHD and improves survival [33].

Chronic GVHD occurs in 20–50% of long-term survivors. Risk factors include older age, prior acute GVHD, use of donor buffy coat infusions, and prior HSV infection. In 20% of cases there is no history of prior acute GVHD. Patients are at risk for developing chronic GVHD from 3 months after transplantation to 6 months after discontinuation of all immunosuppressive therapies. Recent studies show that histologic changes consistent with chronic GVHD may be detected within 60 days of transplant. Common manifestations of GVHD include the sicca syndrome, lichen planus-like skin rash, scleroderma-like skin changes, esophageal and intestinal fibrosis, obstructive lung disease with or without pneumonitis, and elevated alkaline phosphatase with or without hyperbilirubinemia. Immunologic deficiencies including

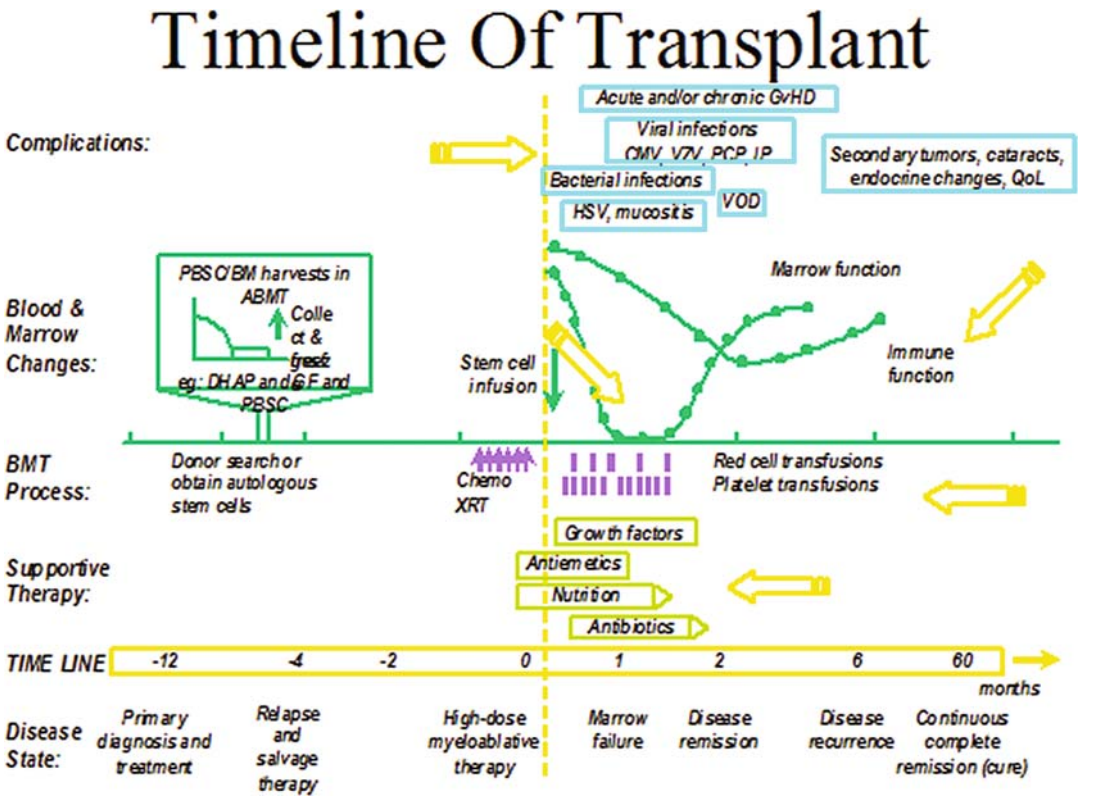

Figure 15.2. Temporal relationship of most common transplant complications.

hypogammaglobulinemia are common, placing patients at increased risk for infections [39].

Patients who undergo stem cell transplantation are at risk for secondary malignancies, with 10–13% incidence at 15-years post-transplant. Malignancies include NHL, myelodysplastic syndrome, skin cancer, head and neck cancers, as well as other solid tumor malignancies. Age and immunosuppressive therapy for chronic GVHD are significantly correlated with the risk of developing a secondary malignancy.

The intensive treatment and prolonged recovery from an allogeneic transplant have profound psychosocial implications for patients and their families. A pre-transplant psychosocial evaluation may help identify individuals who need additional intervention after transplantation. Most long-term survivors, however, report good-to-excellent health and function, with outcomes comparable to long-term cancer survivors who received less-intensive treatments [65,97].

15.5 The Future of SCT

In summary, allogeneic SCT can cure a variety of hematologic malignancies and non-malignant disorders. Even though thousands have had successful allografts, many patients are not cured or die from treatment-related complications. GVHD and disease recurrence remain the main barriers. Strategies to improve the outcome of allografting have focused on combining targeted therapies or adding immunotherapy utilizing vaccines or cellular infusions, particularly of NK cells.

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16.

The Role of Radiosurgery in the Treatment of Bone Localized Cancers

Marsha Haley, Peter C. Gerszten, and Steven A. Burton

16.1 Introduction

Metastatic disease remains a significant source of morbidity for the cancer patient. Approximately 50% of patients diagnosed with cancer will develop metastases, and of these, 70% will develop symptoms [10]. Pain is the most common symptom resulting from bone metastases and is described as severe in more than 50% of hospitalized cancer patients [4].

The spine is the most common site for bony metastatic disease; even with aggressive therapy, 30–70% of patients with cancer have spinal metastatic disease at autopsy. More than half of patients with spinal metastasis have several levels of involvement [37]. Currently, the majority of patients are treated with surgery, medical therapy, radiation therapy, or a combination of modalities. This chapter will focus on the radiotherapeutic modalities used to treat malignant diseases of the bone, with special focus on the use of stereotactic radiosurgery to treat metastatic disease to the spine.

16.2 Historical Background

16.2.1 Discovery and Implementation of External Beam Radiation Therapy

The field of radiation oncology is rooted in Europe, where in the late 1800s Wilhelm Roentgen discovered that X-rays could pass through materials and blacken photographic film. It was quickly realized that radiation produces biological as well as physical effects. Many of the early studies of the biological effects of radiation on tissues were conducted using radioisotopes, radium in particular. Eventually large quantities of radium were placed at a distance from the patient to allow for external beam treatment, called telecurietherapy. This was the birth of external beam radiation therapy, defined as the delivery of ionizing radiation from a source external to the patient for medically therapeutic purposes. Subsequently, higher-energy kilovoltage X-ray generators were produced in an attempt to reproduce telecurietherapy without

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the use of radioactive sources [9]. The prescription for medical external beam radiation is measured in Gray (Gy), defined as 1 Joule per kilogram of absorbed dose [23].

While the kilovoltage units were an exciting development, they had characteristics that made them undesirable for certain treatments. The relatively low energy of the radiation made it difficult to treat deep-seated tumors without significant skin toxicity. In addition, they had a fixed beam – that is, the beam could only be directed at one angle. In the 1950s in Canada, a cobalt source was developed with an average energy of 1.25 million electron volts (much higher than the peak kilovoltage of most existing equipment at that time). A cobalt machine was designed in such a fashion that the source rotated around the patient in the axial plane. This was called isocentric technique and allowed concentration of the dose to the tumor, but spread the dose over a larger volume of normal tissue [31]. Development of cobalt teletherapy units caused the kilovoltage machines to lose popularity.

Even before the cobalt machine was developed, scientists had been experimenting with accelerating particles in evacuated glass tubes. The particles could be accelerated to high velocities by a potential difference in the tube. Eventually, the speed of the particles was increased by placing many tubes in a series [26]. When the particles struck a high atomic number target, high-energy X-ray beams resulted. The first linear accelerator in the world was developed in London in 1952. Four years later, Henry Kaplan at Stanford unveiled the first medical linear accelerator in the Western hemisphere [1] (Fig. 16.1). Kilovoltage machines still had a niche for superficial tumors; however, once linear accelerators with electron capability came on the scene, kilovoltage machines became obsolete [22]. In addition, linear accelerators had several advantages over the cobalt units – the ability to choose different photon and electron energies, superior depth–dose characteristics, and lack of radioactivity. Today, the majority of external beam radiation therapy in developed countries is delivered by linear accelerators.

The modern accelerator continues to evolve from its early ancestors in London and California. Treatment planning was initially done using

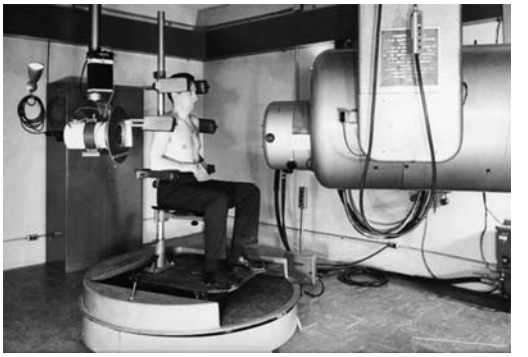

Figure 16.1 Stanford's original linear accelerator. (By permission of Stanford Department of Radiation Oncology.)

X-ray films. In the 1980s CT scanners became more widely available, and this revolutionized radiation treatment planning. The radiation oncologist was now able to see the pertinent anatomy in three dimensions and tailor the treatment beams appropriately. Further development came in the 1990s with intensity-modulated radiation therapy, or IMRT. A full description of IMRT is beyond the scope of the chapter – however, a key feature of IMRT is that it allows further conformation of the beam by varying the intensity across multiple individualized fields. With the addition of high-resolution CT, MRI, and PET, it became possible to delineate tumor volumes with more accuracy. The nature of IMRT requires precise localization of the targets and critical structures so as to avoid missing the tumor site. To avoid that, linear accelerators are increasingly equipped with image-guided radiation therapy, or IGRT. These include imaging of fiducial markers, ultrasound-guided imaging of anatomy, detection of radiofrequency sources, video-based surface tracking, megavoltage CT imaging, and electronic portal imaging devices [34] (Fig. 16.2). Fiducial markers are small markers that have been implanted within the tumor area that are used for position verification throughout the procedure.

16.2.2 Evolution of Radiosurgery

Radiosurgery was developed by the Swedish neurosurgeon Lars Leksell. In 1951 he coined

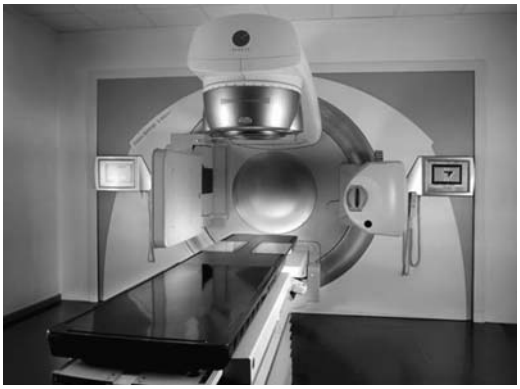

Figure 16.2. The Synergy® unit, a modern linear accelerator. (Courtesy of Elekta.)

this term to describe the delivery of a single high dose of precisely focused radiation to achieve destruction of a small intracranial target through the unopened skull [30]. The Gamma Knife® was the first dedicated stereotactic unit. It was constructed in 1967 by Dr. Leksell and Borje Larsson, who used Cobalt-60 as the energy source. The modern Gamma Knife® has a similar design but houses 201 Cobalt-60 sources that are collimated to focus on a single point to deliver a high dose of radiation. The central axes of all beams intersect with a mechanical precision of 0.3 mm [25]. This machine, by virtue of its design, is used to treat cranial lesions exclusively (Fig. 16.3).

Linear accelerators also have been adapted to deliver stereotactic treatments by changing the shape of the portal, using multileaf collimators, and combining stationary and arcing beams. Accelerator-based stereotactic systems are either pedestal- or couch-mounted. A stereotactic frame is attached to the patient's skull,

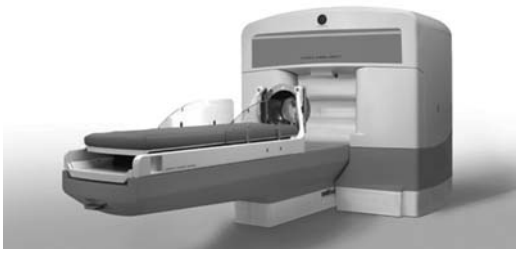

Figure 16.3. Leksell Gamma Knife® unit. (Courtesy of Elekta.)

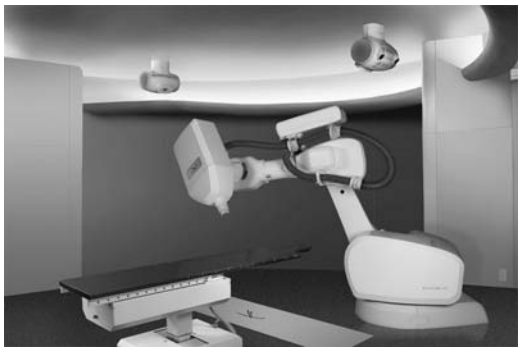

Figure 16.4. Cyberknife™ unit. (Courtesy of Accuray.)

much like the Gamma Knife®, and the frame is mounted on the couch or pedestal. The stereotactic isocenter must coincide with the linac isocenter within 1 mm [24].

The development of Gamma Knife® and linear accelerator-based radiosurgery allowed delivery of highly conformal doses of radiation to the target, while minimizing the dose to the surrounding tissues. There were, however, limitations with these methods. As stated, the Gamma Knife® is only used for cranial sites, and the linear accelerator can only adjust the beam position while utilizing two degrees of rotation with the couch and gantry. To overcome these limitations, Dr. John Adler developed the Cyberknife™, which consisted of a lightweight linear accelerator mounted to a robot with six degrees of rotation. The Cyberknife™ also employed a revolutionary image-guidance system that allowed the use of frameless stereotactic treatment (Fig. 16.4). The first Cyberknife™ prototypes were used in the 1990s; in 2001 the FDA granted clearance for the treatment of extracranial lesions [21].

16.3 Radiobiological Considerations

The critical target of radiation damage is the DNA structure. Radiation causes damage to DNA by liberation of charged particles, which then interact with the DNA molecule to cause damage [15]. If both DNA strands are damaged, the chromatin may break, resulting in cell death

[16]. The rationale of fractionated radiotherapy is rooted in the historical "four Rs" of radiobiology: *R*epair of sublethal damage, *R*eassortment of cells within the cell cycle, *R*epopulation, and *R*eoxygenation. Dividing a single radiotherapeutic dose into a number of fractions spares normal tissues because sublethal damage may be repaired and cells replaced between fractional treatments. Also, dividing the dose increases damage to the tumor because of reoxygenation (oxygen is a potent radiosensitizer) and reassortment of the cells into radiosensitive phases of the cell cycle. In other words, overall treatment time, total dose of radiotherapy, and dose per fraction are important. The dose–response curve for late-responding tissues is more curved than for early responding tissues, so that if fewer and larger fraction sizes are given, late reactions are more severe [19] (Fig. 16.5). This is the reason that in the radiosurgical realm, where the number of fractions is significantly decreased, the dose limitations for critical structures are decreased as well.

The dose of radiation that can be delivered is limited by tissue tolerance. Tissues of children are quite radiosensitive; this includes bone. A dose of 10 Gy in a child can cause death of chondroblasts, and a dose above 20 Gy causes irreversible growth deficit. In adults, radionecrosis can be a serious complication for regions that receive a very high dose of radiation, as is true for the mandible when head or neck cancers are treated by radiation. The humeral and femoral heads may undergo fracture with high-dose treatment, as for sarcomas [18]. Another consideration in treating the bone is the exquisite radiosensitivity of the bone marrow. The risk of hematological toxicity requiring treatment breaks has been shown to be related to the volume of bone marrow subjected to 10 Gy in patients who receive platinum-based chemotherapy [29]. When in the treatment of spinal metastases the number of vertebral bodies subjected to radiation therapy is high, the bone marrow and the hematological profile are affected, especially if there is concurrent chemotherapy.

In the treatment of bone metastases, it is not typically the dose to bone or marrow that is the principal concern. Rather, it is the radiosensitive tissues that are in close proximity to the tumor. When vertebral bodies are treated, the dose that reaches the spinal cord must be taken into consideration. Typically, a 20-cm length of the spinal cord will tolerate a dose of ~45 Gy and a 5–10 cm length will tolerate 50 Gy, provided radiation consists of daily fractions of 2 Gy. At those doses, however, 5% of the patients will have complications such as paralysis by the end of 5 years [11]. Because this is considered unacceptable by most physicians, clinical practice is limited to 45 Gy for any spinal cord volume. Other organs that are critically radiosensitive include the kidneys, small bowel, lungs, and gonads. If a patient presents with disease in an area in which radiation has already been given, this presents a challenge, and radiosurgery is an alternative for these patients.

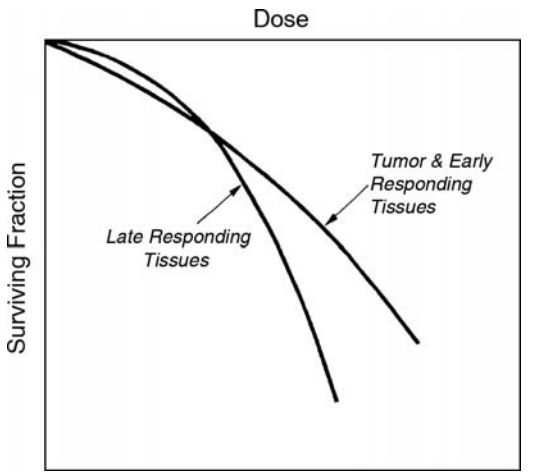

Figure 16.5. Dose–response relationship for late- and early-responding tissues. Reprinted from Hall's Radiobiology for the Radiologist, 6th ed., by permission of Wolters Kluwer Health.

16.4 Current Applications

16.4.1 Clinical Evaluation and Dosing for External Beam Radiation Therapy

Many patients with bone metastases are referred to radiation oncologists for evaluation because radiation therapy results in palliation of more than 73% of patients with bone metastases [12].

At the initial evaluation, the history should include details on the length, quality, and quantity of pain, as well as of alleviating or aggravating factors. Pain scales are helpful in quantifying pain and in assessing response. The physical examination should focus on pain location, anatomical distribution, and neurological deficits. If there is instability and/or if the X-rays show significant cortical destruction, the patient should be referred to a spinal surgeon for evaluation. Further imaging may be needed. Bone scans can detect early functional changes and therefore are more sensitive than X-rays for detecting metastases inasmuch as X-rays require a minimum of 50% trabecular bone destruction for visualization. CT scans are helpful in cases where abnormalities are difficult to evaluate with conventional radiographs. MRI will detect cancellous bone and bone marrow involvement. Because MRI can be viewed in multiple planes, it can indicate nerve or cord compression [32].

Once the necessary information is obtained, a decision needs to be made as to whether the patient is a suitable candidate for external beam therapy. If so, the patient undergoes a treatment planning session on a fluoroscopic simulator or CT scanner. Based on the imaging, field arrangements are planned and calculations are performed with computer assistance (Fig. 16.6).

Many trials have been undertaken to assess the optimal dosage for bone metastases. In 1974 the Radiation Therapy Oncology Group assigned patients with a single metastasis randomly to either a group that received 40.5 Gy in 15 fractions or one that received 20 Gy in 5 fractions. In patients with multiple metastases the doses were 30 Gy in ten fractions, 15 Gy in five fractions, 20 Gy in five fractions, or 25 Gy in five fractions. There was no significant difference in promptness or frequency of pain relief between the different fractionation schemes. A primary site of breast or prostate cancer with an initial pain score of less than 9 was considered positive for prognosis; 90% of patients achieved minimal relief, 83% achieved partial relief, and 54% achieved complete relief, with relief attained in 4 weeks for most patients [36]. Reanalysis of the data in 1985 led to the conclusion that the combination of more fractions with a higher total dose was more effective [3]. In 2003 the Radiation Therapy Oncology Group reported on a phase III trial that compared a dose of 8 Gy in one fraction with one of 30 Gy in ten fractions in patients with bone metastases from breast and prostate cancer. The results, published in 2005, showed

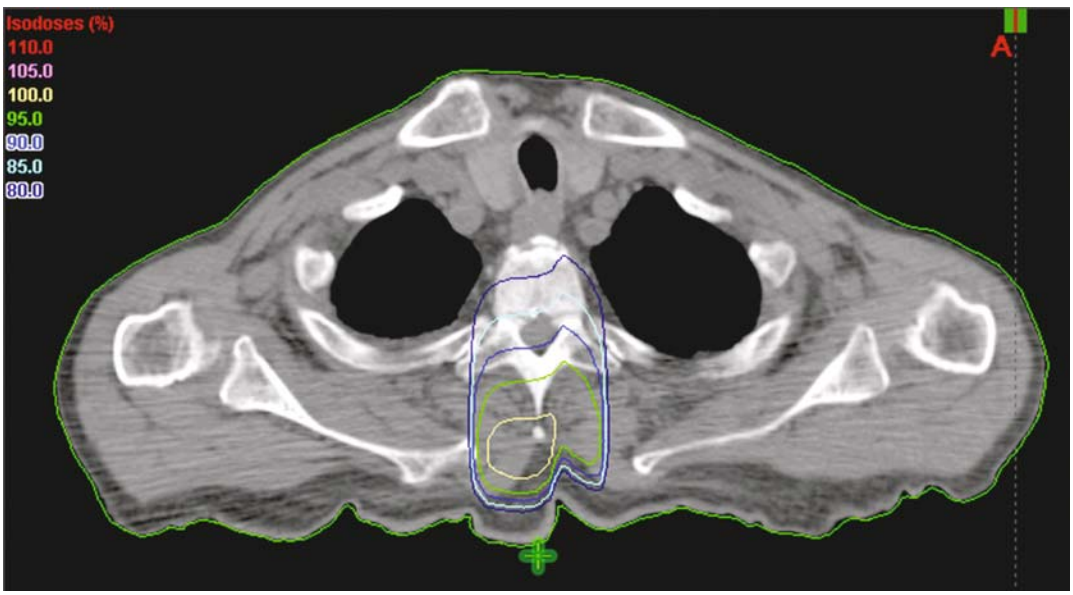

Figure 16.6. External beam radiation treatment plan. (Courtesy of University of Pittsburgh Cancer Institute.)

that the rates of acute toxicity were significantly higher in the 30-Gy group but the need for retreatment was greater for the 8-Gy group [20]. A 2007 meta-analysis with data from 16 randomized trials, beginning in 1986, agreed with the earlier studies that pain relief was essentially the same, regardless of the fractionation schedule. However, single-fraction treatment was associated with a higher retreatment rate [8].

16.4.2 Clinical Evaluation and Dosing for Stereotactic Radiosurgery

In radiotherapy, proper localization is needed to deliver the prescribed dose to the diseased region without damage to the surrounding normal tissue. In radiosurgery, proper localization is even more critical because of the high doses delivered per fraction and the rapid fall-off of radiation outside of the target area. Precise localization depends on two principal factors: rigorous specifications and high quality of the equipment, and target immobilization. Immobilization devices are often used to keep the patient still during treatment. Successful external beam radiotherapy depends on a daily setup error that is within several millimeters. For radiosurgery, however, setup accuracy must be within 1 mm. Because this cannot be accomplished with standard radiotherapeutic immobilization devices, the invasive frame is commonly used for intracranial radiosurgery. When extracranial radiosurgery was first employed, invasive skeletal fixation was used both above and below the involved vertebral segments [2].

Even with the use of IMRT, target immobilization and localization cannot be achieved to the degree that would permit treatment in a single fraction [35]. In 2001 investigators at the Universities of Iowa and Florida published results of an optically tracked ultrasonography unit that could be registered to a linear accelerator coordinate system. They applied this technique to patients with localized metastatic spinal disease with a linear accelerator, by programming the system to track structures in real time and update position based on predetermined virtual CT volumes [33]. At the same time physicians at Stanford University were using the Cyberknife™ to track structures with a fixed relationship to the tumor, such as vertebral bodies or fiducial markers [21]. Since that time, multiple studies have shown that frameless stereotactic radiosurgery is safe and efficacious for treating spinal tumors [2].

Clinicians at the University of Pittsburgh have very extensive experience with extracranial radiosurgery [13]. At our institution, candidates for spinal radiosurgery are evaluated in a multidisciplinary setting by a radiation oncologist and a neurosurgeon. Lesions that are appropriate for spinal sterotactic radiosurgery include well-circumscribed lesions, lesions with minimal spinal cord compromise, radioresistant lesions that would benefit from a radiosurgical boost, residual tumor after surgery, previously irradiated lesions, recurrent surgical lesions, lesions requiring difficult surgical approaches, and lesions in patients with a short life expectancy or significant medical comorbidities that preclude open surgical intervention. Exclusion criteria for spine radiosurgery include overt spinal instability, neurologic deficit from bony compression of neural structures, cauda equina syndrome, and myelopathy [35].

The first step in CyberKnife™ spinal radiosurgery treatment is similar to the CT treatment planning for external beam radiotherapy. First one must consider the anatomical location and plan the type of target localization device. The Cyberknife™ can track implanted fiducials, as well as anatomical reference points with the aid of the Xsight™ Spine Tracking System [28]. Patients with cervical lesions are fitted with a noninvasive molded Aquaplast facemask (Aquaplast Corp., Wyckoff, NJ) which immobilizes the head and neck. CT slices of 1.25 mm are then obtained, proceeding from the top of the skull to the bottom of the cervical spine. If the patient is to undergo open surgical manipulation in the thoracolumbar region prior to radiosurgery, fiducial markers can be inserted at that time; the remainder undergo fluoroscopically guided percutaneous insertion of 4–6 gold fiducial markers (Alpha-Omega Services, Inc., Bellflower, CA) that are placed into the pedicles immediately adjacent to the lesion to be treated. This is done with a standard Jamshidi Bone Marrow Biopsy Needle (Allegiance Healthcare

Figure 16.7. Fiducial markers. (Reprinted from Szeifert et al. [35, pp. 340–358], by permission of S. Karger AG Medical and Scientific Publishers.)

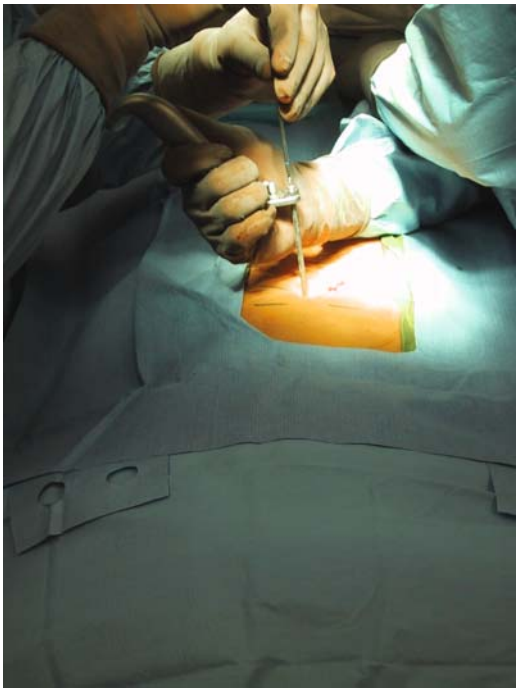

Figure 16.8. Placement of fiducial markers. (Reprinted from Szeifert et al. [35, pp. 340–358], by permission of S. Karger AG Medical and Scientific Publishers.)

Corp., McGraw Park, IL) (Figs. 16.7 and 16.8) [14]. The fiducial placement procedure is performed in the operating room, in an outpatient setting, and precedes the planning CT. The patient is placed in a supine position in a conformal vacuum cradle both during CT imaging and during treatment.

The second component of the CyberKnife™ treatment involves the development of the computerized treatment plan for radiosurgery. The planning CT data are transferred to the Accuray planning system. The radiation oncologist and neurosurgeon contour the tumor and the structures, such as the spinal cord, where the dose must be restricted. Initially doses were chosen on the basis of intracranial radiosurgery doses; however, the dose is best based on experience in the treating institution and retrospective data, keeping in mind the limitation imposed by the maximum dose that can be tolerated by the spinal cord. Typical prescription doses are 16–20 Gy, although doses can be adjusted, depending on the clinical situation. The prescribed dose is the 80% isodose line; as a result the tumor center receives a dose greater than the prescription dose [13]. Once the contours are completed and the prescription written, the medical physicist develops a plan to meet the dose specifications outlined by the physician (Fig. 16.9). The physicist also identifies the location of the fiducial markers or reference anatomy in relationship to the target.

The third component of the CyberKnife™ treatment is delivery of the actual treatment. Spinal treatments are performed using a single-fraction technique. Treatments are performed by a radiation therapist with special training in radiosurgery. The patients are appropriately immobilized with the aid of the immobilization device and are then placed on the CyberKnife™ treatment couch. In most cases preoperative analgesia or sedation is not required; however, in rare situations patients may require premedication or even conscious sedation. The procedure itself is not painful; patients may require medication due to the length of the procedure and because they have to remain immobilized. With the aid of X-ray cameras, near real-time digital radiographic images of the implanted fiducial markers are obtained during treatment. The planning system allows the machine to know the relationship of these fiducials to the target vertebral body and makes it possible to adjust

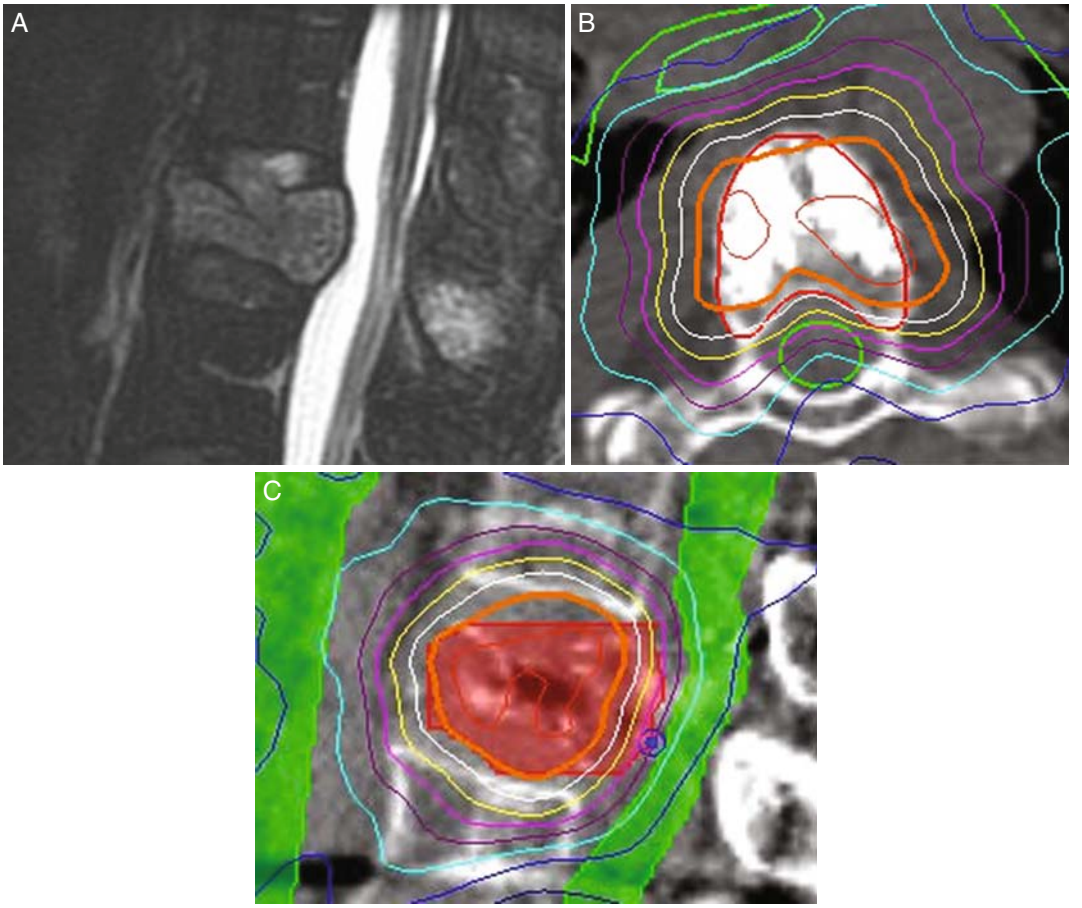

Figure 16.9. A case example of an L1 thyroid carcinoma metastasis in a 75-year-old man. The patient, treated previously with conventional external beam irradiation, presented with pain recurrence and radiography indicated his tumor had progressed. A T2 sagittal MRI shows a pathologic L1 compression fracture with spinal canal compromise (**A**). The patient underwent percutaneous transpedicular methylmethacrylate placement and biopsy. He subsequently received radiosurgery to the lesion with a maximum prescription dose of 22.5 Gy delivered in a single session. The maximum dose to the spinal cord was 8.5 Gy (**B** and **C**). (Courtesy of University of Pittsburgh Cancer Institute.)

the table and machine position accordingly (Fig. 16.10). When Xsight^{TM} is used, the same procedure is followed, except that the machine adjusts the position on the basis of the bony landmarks. Closed-circuit television is used to observe the patient during the treatment, and there is a two-way microphone for the therapist and patient to communicate. Treatment typically lasts 30–60 min and the patient is discharged at the end of treatment. The patient returns to the multidisciplinary clinic after 1 month for physical examination, evaluation of pain response, and toxicity.

At the University of Pittsburgh, spinal radiosurgery has been highly effective in causing decreased pain in patients with spinal metastases. This group recently published a prospective longitudinal cohort study of 500 cases of spinal metastases treated with stereotactic radiosurgery. Treatment plans were customized according to the patient's anatomy and previous radiation. Tumor doses ranged from 12.5 Gy to 25 Gy, with a mean dose of 20 Gy. Long-term improvement of pain occurred in 86% of cases and radiographic tumor control was achieved in 88%. Follow-up for 21 months so far has not

Figure 16.10. Patient localization and treatment on the Cyberknife™ unit. (Courtesy of Accuray.)

revealed neurological toxicities due to treatment [13].

16.5 Conclusion/Future Directions

Advances in care notwithstanding, bony metastatic disease remains a significant source of morbidity for the cancer patient. Standard treatment options for spinal metastases include external beam radiotherapy, radionuclide therapy, systemic chemo/hormonal therapy, and surgical intervention. A major goal of local radiation therapy in the treatment of spinal tumors is palliation of pain [27]. The concept of hypofractionated radiotherapy for symptomatic bone metastases originated 30 years ago and has been studied in multiple clinical trials. Single-fraction radiotherapy was found to be as efficacious as multiple-fraction radiotherapy. However, patients who received single-fraction radiotherapy had a higher rate of repeat radiation [8]. Most trials used 8 Gy in a single fraction; however, no trials specifically evaluated spinal metastases. Delivering a dose in a single large fraction is theoretically more effective for relatively radioresistant tumors, but external beam radiotherapy delivered in a single large dose is not desirable because of the risk of late tissue damage to the spinal cord [17]. Radiosurgery makes it possible to deliver a high single-fraction dose to the tumor while sparing adjacent normal tissue.

Advances in technology have allowed the delivery of highly conformal doses of radiation delivered in a single fraction. The University of Pittsburgh began using the Cyberknife™ in 2001 for the treatment of spinal metastases, and since that time more than 1000 cases have been treated. Initially, spinal stereotactic radiosurgery (SRS) was used for patients who had been previously treated with radiation therapy. SRS is however emerging as a primary treatment modality for spinal metastatic disease.

Another treatment gaining popularity is stereotactic radiotherapy, which is a compromise between external beam radiotherapy and stereotactic radiosurgery. The technical advances in the modern linear accelerator allow more precise target definition and conformality, making hypofractionation more feasible. In 2004 Chang et al. at MD Anderson Cancer Center published phase 1 results of near-simultaneous computed tomographic image-guided stereotactic radiotherapy for treating spinal metastases to a dose of 30 Gy in five fractions. The technique was found to be feasible and highly accurate in the 15 patients studied [6]. In 2005, Yamada et al. at Memorial Sloan-Kettering Cancer Center published results of 35 patients treated in five fractions with intensity-modulated stereotactic radiotherapy. The previously irradiated patients received a median dose of 20 Gy, and the unirradiated patients received 70 Gy. The results were similar to those obtained in the MD Anderson study [38]. Chang et al. recently published the results of a phase I/II study of stereotactic body radiotherapy for spinal metastases. In the course of the study, the protocol was amended from 30 Gy in five fractions to 27 Gy in three fractions, thus decreasing treatment time. This approach was well-tolerated with no cases of grade 3 or 4 neurological toxicity, and the actuarial rate of radiologic tumor control was 84% at 1 year [7].

In conclusion, current radiotherapy remains an effective method to palliate for spinal metastases with low toxicity, irrespective of the mode of delivery. Surgeons have now joined

the radiation oncology team and are an important part of the radiosurgery process. This inter-specialty cooperation has led to significant advances in the fields of radiotherapy and neurosurgery and, with ever-increasing dialogue, the potential of new technology will be more widely explored [5].

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