Bone Metastasis and Molecular Mechanisms

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Bone Metastasis and Molecular Mechanisms

Pathophysiology

Edited by

Gurmit Singh
Director of Research, Juravinski Cancer Centre/
Professor of Pathology & Molecular Medicine,
McMaster University, Hamilton, Ontario, Canada

and

William Orr
Chair, Department of Pathology, University of Manitoba,
Winnipeg, Manitoba, Canada

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Dedicated to:

Bone Metastasis Researchers and Cancer Patients.

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CONTRIBUTORS

EDITORS
Gurmit Singh
Director of Research
Juravinski Cancer Centre
Professor of Pathology and Molecular Medicine
McMaster University
Hamilton, Ontario, Canada

William F. Orr
Chair of Pathology
Health Sciences Center
University of Manitoba
Winnipeg, Manitoba, Canada

CONTRIBUTORS
Robin L. Anderson
Head, Cancer Biology Laboratory
Peter MacCallum Cancer Institute
East Melbourne, Victoria, Australia

Nicholas A. Athanasou
Departments of Surgery and Pathology
Nuffield Department of Orthopaedic Surgery
University of Oxford
Oxford, UK

Andrew Belch
University of Alberta and Cross Cancer Institute
Alberta, Canada

Paolo Bianco
Dipartmeento di Medicina Sperimentale e Patologia
Universita "La Sapeinza"
Parco Scientifico Biomedico San Raffaele
Rome, Italy

Jeroen Buijs
Department of Endocrinology
Leiden University Medical Center
Leiden, Netherlands

Wilhelmina C.M Duivenvoorden
Northwestern Ontario Regional Cancer Centre
Thunder Bay, Ontario, Canada

Bedrick L. Eckhardt
Peter MacCallum Cancer Centre
East Melbourne, Victoria, Australia

Thomas H. Hassard
Department of Community Health Science
University of Manitoba
Winnipeg, Manitoba, Canada

Paul J. Kostenuik
Research Scientist
Amgen Inc.
Thousand Oaks, California, USA

Mary Mackenzie
Medical Oncology
Juravinski Cancer Centre
Hamilton, Ontario, Canada

Pierre P. Major
Medical Oncology
Juravinski Cancer Centre
Hamilton, Ontario, Canada

Andrew P. Mazar
Attenuon, LLC
San Diego, California, USA

Belinda S. Parker
Peter MacCallum Cancer Center
East Melbourne, Victoria, Australia

Linda M. Pilarski
University of Alberta and Cross Cancer Institute
Alberta, Canada

Patrick M. Pilarski
University of Alberta and Cross Cancer Institute
Alberta, Canada

Shafaat A. Rabbani
Departments of Physiology and Medicine
Royal Victoria Hospital
McGill University Health Centre
Montreal, Quebec, Canada

Martin Reed
Department of Radiology
Health Sciences Center
Winnipeg, Manitoba, Canada

Tony Reiman
University of Alberta and Cross Cancer Institute
Alberta, Canada

Mara Riminucci
Parco Scientifico Biomedico San Raffaele
Rome Italy
Departmento di Medicina Sperimentale
Universita dell'Aquila
L'Aquila, Italy

Pamela Gehron Robey
Craniofacial and Skeletal Diseases Branch
National Institute of Dental and Craniofacial Research
National Institutes of Health
Department of Health and Human Services
Bethesda, Maryland, USA

Eric Seidlitz
Department of Research
Juravinski Cancer Centre
Hamilton, Ontario, Canada

Julie A. Sharp
Department of Surgery
University of Melbourne
St. Vincent's Institute of Medical Research
Fitzroy, Australia

Jonathan C. Sharp
Institute for Biodiagnostics
National Research Council of Canada
Winnipeg, Manitoba, Canada

Nicholas Shukeir
Departments of Physiology and Medicine
Royal Victoria Hospital
McGill University Health Centre
Montreal, Quebec, Canada

Sadmeet Singh
Department of Research
Juravinski Cancer Centre
Hamilton, Ontario, Canada

James A. Thliveris
Department of Human Anatomy and Cell Science
University of Manitoba
Winnipeg, Manitoba, Canada

Erik W. Thompson
University of Melbourne
Department of Surgery
St. Vincent's Institute of Medical Research
Bernard O'Brien Institute of Microsurgery
St. Vincent's Hospital
Fitzroy, Australia

Gabri Van Der Pluijm
Leiden University Medical Center
Department of Endocrinology
Leiden, Netherlands

Snezana Vikmirovic-Popovic
Department of Research
Juravinski Cancer Centre
Hamilton, Ontario, Canada

Michael H. Weber
Department of Pathology
University of Manitoba
Institute for Biodiagnostics
National Research Council of Canada
Winnipeg, Manitoba, Canada

PREFACE

Bone is a frequent site for metastasis in patients with carcinomas of the breast, lung and prostate and hematopoietic malignancies. They result in significant clinical morbidity and are usually related to extensive tumor-associated osteolysis. Despite the magnitude of this problem, the current therapies have had variable success. The tumor cells relationship with host tissue is a major factor in their ability to develop into macroscopic metastases. The "seed" and "soil" hypothesis of metastasis was proposed in Paget's paper of 1889 discussing the distribution of metastasis in breast cancer. Unraveling the molecular mechanisms governing the interaction between the host microenvironment and tumour cell is key to the development of new therapies to deal with metastases.

This multi-authored book focuses specially on the pathophysiology of bone metastases. The writers are all active investigators with research programs on bone metastasis and leaders in the field. They have reviewed the current literature in their areas of research. The first chapter by Dr. Robin Anderson's group discusses various models of breast cancer metastasis. Chapter 2 from Dr. Erik Thompson's group is focused on the quantitation aspects of bone metastasis. Chapters 3 and 4 from Dr. Gurmit Singh and Dr. Bill Orr's group have outlined comparative morphometric techniques to study bone metastasis and clinical modalities for the diagnosis of bone metastasis. Chapter 5, 6, 7 and 8 have a theme on the microenvironment and associated pathophysiologies of bone metastasis. In chapter 5, Dr. Pamela Robey has outlined the metastasis in the bone marrow environment. Dr. Athanasou evaluates the role of tumor-associated macrophages in chapter 6. The relationship between malignant cells and the bone macroenvironment is discussed in chapter 7 by Dr. Linda Pilarski's group. Networking via

angiogenesis and lymphangiogenesis is highlighted in chapter 8 by Dr. Gabri Van Der Pluijm's group.

Chapters 9, 10, 11, 12 and 13 relate to key areas in the development of new therapies for bone metastasis. In chapter 9, Dr. Shafaat Rabbani's group has outlined an approach to developing novel therapeutic intervention using prostate cancer models. In chapter 10, Dr. Gurmit Singh's group provides a unique opportunity of using osteotropic agents in the treatment of bone metastasis. Dr. Paul Kostenuik discusses the role of OPG in pathobiology of osteolysis and potential use as a therapeutic agent. Chapter 12 discusses the role of matrix metalloproteinases in bone metastasis and potential therapeutic targets. Finally in chapter 13 Drs. Pierre Major and Mary Mackenzie provide a rational for the use of bisphosphonates in bone metastasis.

This is a comprehensive treatise to the subject of bone metastasis as it pertains to the pathophysiology and mechanisms of bone metastasis. The authors and editors hope this book will help in furthering fundamental research in bone metastasis.

The editors would like to thank all the contributing authors and congratulate them for their scholarly efforts in the preparation of these chapters, which have summarized current literature in their field. We would also like to thank Dr. Richard Ablin and Wen Jiang for inviting us to edit a volume on bone metastasis for the book series on Cancer Metastasis: Biology and Treatment. We would like to acknowledge assistance from the Publishing Editor, Dr. Cristina Miranda Alves dos Santos and her assistant Ms. Melania Ruiz Esparza from Kluwer press. Finally the support and assistance of Mrs. Pauline Hiltz and Ms. Heather Blackborow is greatly appreciated.

Professor Gurmit Singh

Chapter 1

MODELS OF BREAST CANCER METASTASIS TO BONE: CHARACTERIZATION OF A CLINICALLY RELEVANT MODEL

Belinda S. Parker, Bedrich L. Eckhardt and Robin L. Anderson
Peter MacCallum Cancer Centre, St. Andrews Place, East Melbourne, Victoria, Australia

1. BREAST CANCER: THE CLINICAL PROBLEM

Breast cancer metastasis to the skeleton is a severe clinical problem that occurs in 70% of patients with advanced disease. Bone is also a frequent site of metastasis of some other cancers, including prostate. Bone metastases in breast cancer are predominantly osteolytic due to the resorption of bone following excessive activation of osteoclasts. This leads to major clinical problems including severe pain, hypercalcemia, bone fractures and nerve-compression syndromes (1).

When diagnosed early, breast cancer can be treated successfully, but the advanced disease is difficult to control and chances of survival are much reduced. Treatment of patients with bone involvement is essentially palliative, aiming primarily to minimize pain and other debilitating clinical symptoms, rather than to affect a cure. Due to the severe morbidity of this disease, there is an urgent need for new treatment options. Bone metastases are difficult to treat for several reasons. First, invasion and release of tumor cells into the circulation has often occurred before detection and removal of the primary tumor. Second, the heterogeneity of the primary cancer leads to difficulties in identifying factors that are predictive of metastasis and therefore potential targets for therapy (2). Finally, there is a lack of an understanding of the molecular interactions that take place in the bone

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microenvironment. The search for the best therapeutic targets to block metastasis to bone is hampered by the lack of clinically relevant *in vivo* models of breast cancer metastasis. Identification of genes that are clinically relevant in metastasis to bone is required to shift therapy from palliative to specific and effective treatments. The next section will cover what is known about the steps involved in the metastatic cascade and which of these steps are potential targets for preventing the growth of metastases in bone.

2. MECHANISMS AND POTENTIAL THERAPEUTIC TARGETS FOR METASTASIS TO BONE

The metastatic cascade begins with growth and invasion of the primary tumor and progresses to the formation of macrometastases at distant sites. It is evident that this process involves interactions between the tumor cells, the surrounding stroma and the blood vessels that provide factors essential both for tumor growth and for dissemination of tumor cells (3). Interactions with the stroma are required for the survival and growth of breast cancer cells at specific secondary sites, such as bone. A number of theories have been proposed to explain the increased colonization of tumor cells at specific secondary sites. These theories, including Paget's "seed and soil" hypothesis, the "circulation" theory and the "homing" theory, have been extensively reviewed in the past (4, 5) and will not be discussed here. Several studies have demonstrated a contribution by all three theories, in that the release of cells into the circulation and their arrest may be random or in some cases controlled by a chemotactic homing mechanism (6) but the attachment, survival and growth into macrometastases at secondary sites is specific to stromal interactions within that organ.

Steps involved in metastasis include growth of the primary tumor, loss of cell-cell adhesion, invasion into surrounding stroma, intravasation into the circulation and arrest at a secondary site, either randomly or by specific interaction with vascular endothelial cells. Tumor cells then extravasate from vessels, form micrometastases and later macrometastases if there is a suitable microenvironment (7, 8).

Recent *in vivo* imaging studies have suggested that cells within the circulation extravasate efficiently and somewhat randomly at secondary sites. The rate-limiting step was proposed to be the formation of macrometastatic lesions, with the majority of cells never achieving this step (9). Most cells that extravasate into a secondary tissue die or remain dormant (10). However, a few tumor cells initiate favorable interactions with the

stroma, promoting their survival and growth. It is of great interest to understand the specific molecular characteristics that allow these few cells to initiate the process of tumor growth and bone resorption, a process termed the "vicious cycle" (11). Factors released by tumor cells stimulate osteoclast activation either directly or through osteoblasts leading to bone lysis and release of tumor growth stimulating factors from the bone matrix.

What is the difference between the gene expression profiles of cells that remain dormant and those that can progress to form macrometastatic lesions? It has been postulated that the latter may have the ability to escape immune recognition by expressing a subset of surface proteins that are also expressed by normal cells in the bone environment (12). Could this explain why many of the cells do not die, but remain dormant? Alternatively, could the niche within the bone marrow where a tumor cell lodges after extravasation dictate its future growth capacity? The ability of the tumor cells to interact with bone stromal cells to stimulate angiogenesis is likely to be another important regulator of bone metastasis.

To date, there are few therapies available to block the growth of bone metastases (11, 13). Since the rate-limiting step is likely to be growth at the secondary site, it may be more logical to target this step rather than the initial release of tumor cells into the circulation. The most attractive therapies are those that prevent tumor cell attachment and communication with stromal cells in bone, or that prevent activation of bone-derived growth factors required for bone resorption or that inhibit angiogenesis. Thus, new therapies need to be directed at specific targets in the cell. The rational way to identify these therapeutic agents is to determine the molecular mechanisms that provide tumor cells with the ability to initiate bone destruction. This approach requires the utilization and further development of good models of bone metastasis. One class of compounds that have been shown to interfere with bone resorption is the bisphosphonates. As well as blocking bone resorption, the bisphosphonates inhibit tumor cell attachment to bone, inhibit activity of MMPs and promote apoptosis of tumor cells (14, 15). It is important to determine the specific mechanism of action of the bisphosphonates to aid in the search for analogs with enhanced activity. Clinically relevant models of metastasis to bone are needed for complete analysis of therapeutic potential. These models can also be used to measure the efficacy of these new therapies in combination with other anti-cancer agents, especially since patient survival is not always improved by bisphosphonate treatment alone (14, 16, 17). However, treatment with bisphosphonates before the onset of bone metastases has been shown to reduce metastatic onset and prolong patient survival (18).

Another promising therapy that needs to be tested in more clinically relevant models of malignant bone disease is osteoprotegerin (OPG), a

soluble decoy receptor for RANK ligand, which is a TNF-related protein released by osteoblasts to trigger activation of osteoclasts. Preclinical studies indicate that OPG is effective in inhibition of osteolysis caused by tumor cells (19, 20).

The interactions between tumor cells and bone stroma that initiate the cycle of bone destruction and tumor growth are critical for understanding the bone metastatic process (21). Although there have been studies of tumor cell growth in the bone environment (22, 23), the biology/pathogenesis of bone metastasis is still not well understood due to the lack of models that mimic the entire process of breast cancer metastasis.

It is likely that expression of a number of genes in the primary tumor and/or in the bone microenvironment will be important for the development of osteolytic bone metastases (7, 8, 11, 23). Those already identified as potentially important include parathyroid hormone-related protein (PTHrP) (a major contributor to bone resorption that will be discussed in more detail later in the chapter), interleukin-11 (IL-11) and IL-6, which have been shown to stimulate bone resorption and may (like PTHrP) induce osteolysis (24), calcitonin receptors, the bone proteins osteopontin and bone sialoprotein (25), tumor necrosis factor-α (TNFα) (26), transforming growth factor-β (TGF-β) (27), prostaglandins (28), receptor activator of NF-κB ligand, (RANKL) (29), macrophage-colony stimulating factor (M-CSF) (29, 30) and human platelet derived growth factor-BB (hPDGF-BB) (31). A number of other genes have been shown to be involved in the metastatic process, but are not specific to bone metastasis such as E-cadherin, VEGF, uPA (32), αvβ3 integrin (33), maspin (34), matrix metalloproteinases (MMPs) (35) and other proteases that may aid in invasion. There are now over twenty MMPs identified and it is likely that specific MMPs will be required for local invasion and release of cells from the primary tumor whilst others are active at different sites of metastasis. This remains an important aspect of metastasis that has not been thoroughly investigated.

Although many of the above mentioned candidate genes are differentially expressed in secondary bone tumors compared to primary tumors, a single accurate predictor to identify patients who will develop bone disease is still lacking.

3. MODELS OF METASTASIS TO BONE

An obstacle for research on bone metastasis is the lack of patient samples, since bone metastatic lesions are rarely removed surgically. In cases where the lesion is removed, access to a sample of the matching primary tumor is unlikely to be available due to the time delay between

primary tumor onset and metastatic occurrence. It is therefore very difficult to compare primary and metastatic lesions from the same patient. Archival samples are useful for genetic screens and *in situ* hybridisation or immunohistochemical techniques for expression analysis of selected genes. However, if a gene is found to have altered expression, it is very difficult to determine its function, whether it promotes or suppresses metastasis or whether it is downstream from the effector gene. It is also difficult to analyze the effectiveness of specific treatments to target the activity of the gene. Obviously the preferred approach is a model that can be utilized for genetic screens and is amenable for functional studies. Good models are not only important for a study of breast cancer pathogenesis and gene function but also for assessing the activity and potential of new therapies prior to patient administration.

Many current animal models of metastasis are not ideal for functional studies or drug testing since they do not encompass the whole metastatic process from primary tumor formation to growth of bone metastatic lesions. Very few metastasis models spontaneously metastasize to bone, most only mimic the latter stages of bone metastasis. It must therefore be questioned whether the results obtained from these experimental metastasis models are relevant to the complete process in breast cancer patients. However, despite their limitations, the commonly used experimental models have yielded much of our current knowledge of metastasis to bone, as summarized below.

3.1 Xenograft Metastasis Models

Xenograft models have been used extensively for studying bone metastasis *in vivo*, and have been useful in determining the genes from both tumor epithelium and bone stroma that regulate bone resorption. These models involve injection of human tumor cells into immunocompromised mice. Common avenues for injection are subcutaneous sites, the mammary gland, directly into bone, the left ventricle of the heart and the tail vein.

Subcutaneous or orthotopic injections of human breast cancer cells into the mammary fat pad of immunocompromised mice have not been of value in the study of bone metastasis. Injection of tumor cells into these sites leads to growth of primary tumors that rarely metastasize except to draining lymph nodes and occasionally to lung. However, it has been reported recently that MDA-MB-435 human breast tumor cells can be isolated from lung and bone of SCID mice bearing a mammary fat pad tumor that is excised 8 weeks after tumor cell inoculation (36).

The injection of cells directly into bone (37) lacks both formation of a primary tumor and the process of entry and exit from the circulation, and it is also likely that such an injection will cause injury at the site of injection,

leading to a local inflammatory response. However, this route of injection is useful for the investigation of tumor growth in the bone environment.

Tail vein injections result in lung metastases (38) but rarely lead to the formation of bone metastases. As with the mammary fat pad injections, this may be due to the rapid progression of tumor growth in the lungs of these mice leading to death prior to skeletal involvement, or due to the lung being the first capillary bed for arrest of cells. However, bone seeking MDA-MB-231 clones that have been selected *in vivo* for preferential dissemination to bone, expand and form osteolytic lesions after tail vein injection (39).

The most common model that has been utilized for analysis of bone metastases in breast cancer is injection of MDA-MB-231 human breast cancer cells into the left ventricle of the heart of immunocompromised mice (40). The cells metastasize predominantly to bone and occasionally to brain, ovary and adrenal glands (41). The MDA-MB-231 intracardiac model triggers osteolytic damage in bone, which correlates with the extent of tumor burden (42). This model has enabled numerous studies of the role of specific genes in breast cancer growth in the bone environment. By multiple cycles of injection and recovery of cells from bone, Yoneda and colleagues (43) selected bone seeking clones that retained a similar tumorigenicity to the parental cell line yet metastasized specifically to bone. These clones formed larger osteolytic lesions and expressed increased amounts of PTHrP compared to the parental line.

Although the intracardiac model has provided valuable information on the interactions of tumor cells with bone stroma and the factors involved in osteolysis, there are disadvantages in using this model to study metastatic breast cancer. Three of these disadvantages are:

1. the lack of a primary tumor
2. the introduction of a bolus of circulating cells
3. the use of mice lacking a competent immune system.

In a model lacking a primary tumor, it is difficult to ascertain if the gene expression changes that are found in experimental bone metastases will be relevant when looking at the metastatic process as a whole. It is not possible to show that a gene that seems to be important for secondary tumor growth and/or bone resorption is also expressed in the primary tumor and if it is, whether its expression correlates with the incidence of distant metastasis and hence patient survival. Also, analysis of the processes of migration and invasion in tissue culture or in model systems where no primary tumors are formed, ignores the contribution of tumor-associated stromal cells to motility and invasion of tumor cells from the primary site.

In the pathogenesis of breast cancer metastasis, cells are released from the primary site and enter the circulation in small numbers over a long period of time. It is not known what differences a large bolus of cells will cause, but

it can be hypothesized that cell migration and arrest in distant capillary beds may be influenced. It must also be recognized that the tissue culture cells used for intracardiac injection have not been adapted to *in vivo* growth and selection prior to release into the vasculature.

The immune system can modulate tumor growth and metastasis in both a positive and negative way. Inflammatory cells and various cytokines have been shown to promote metastasis. For example, tumor associated macrophages (TAMs) have a role in progression at both primary and secondary tumor sites. Recent studies have indicated that TAMs promote breast tumor growth (44). There is also a role for cytokines in osteoclastic bone resorption as indicated recently by the finding that chemokines, macrophage inflammatory protein 1-α (MIP-1α) and -β (MIP-1β), are present in bone marrow of patients with active myeloma bone disease and increase osteoclast formation (45, 46). Belonging to the RANTES family, these chemokines act as chemoattractants and activators of monocytes, from which osteoclasts are derived.

Other cells of the immune system, such as cytotoxic T lymphocytes (CTL) and natural killer (NK) cells recognize and kill tumor cells (47, 48). CTL are substantially depleted in nude and SCID mice, thus allowing growth of allogeneic tumor cells. In syngeneic hosts, tumor cells have been reported to undergo changes that allow their escape from immune surveillance. Loss or alteration of the MHC class 1 phenotype will provide a survival advantage to cells, allowing them to escape recognition and killing by CTL and NK cells and consequently develop as metastases (49, 50). A recent study investigated the MHC class 1 phenotype in disseminated tumor cells in bone marrow of patient samples and found a positive association between down regulation of MHC class 1 and unfavorable outcome (49). Thus the loss of MHC class 1 expression may play a role in allowing tumor cells to form bone metastatic lesions, such phenotypic differences thereby defining the cells that can form metastatic lesions. In the absence of a competent immune system, discrimination between cells based on MHC class 1 expression does not occur. Additionally, it has been reported that cells may express specific proteins on their surface to escape host immune surveillance (12, 51-53).

Whilst metastasis models utilizing immunodeficient mice do not consider these important aspects of metastasis, they remain the only way of studying human tumor growth and metastasis in mice.

3.2 Transgenic Mice

Transgenic models of breast cancer express oncogenes specifically targeted to the mammary gland by use of mammary specific promoters such

as whey acidic protein (WAP), rat prostatic steroid binding protein (C(3)1) (54) and mouse mammary tumor virus (MMTV) (55). Transgenic models have considerable advantages over transplantable models in that tumor initiation closely resembles the early events in human cancer. Transgenic mice enable questions to be asked about the contribution of specific genes to tumorigenesis in mammary epithelium. The WAP-TAg (WAP-Simian Virus (SV) 40 T antigen) oncogenic mice are a useful background model of tumor initiation, developing mammary tumors with 100% frequency, and can be used for crossing to transgenic mice bearing a gene of interest to determine its role in tumor progression. An example of such a cross is with WAP-maspin transgenic mice that revealed the role of maspin in inhibiting tumor growth *in* vivo (56). The MMTV promoter system has been used to demonstrate that expression of a number of genes, including the human oncogenes myc (57) and Wnt-1 (58), can cause mammary cancer in mice, as in humans. A similar result was found with the mammary-specific knockout of the tumor suppressor gene BRCA1 (59). However, whilst transgenic models are valuable for studies of both mammary development and the molecular pathways of mammary cancer initiation and progression, no models exist that encompass metastasis to bone. When genes important for metastasis to bone have been identified, it will be possible to return to these transgenic models and cross the mice to strains bearing mammary gland specific expression of one of these genes, thus generating a transgenic model of breast cancer metastasis to bone.

4. PARATHYROID HORMONE RELATED PROTEIN (PTHRP) AND BONE METASTASIS

Studies, largely using the MDA-MB-231 intracardiac model, have found a number of genes that are altered in breast tumors and that appear to have a role in breast cancer progression. One gene that has received considerable attention due to its role in bone biology is PTHrP (60). PTHrP was originally identified as a causal agent of humoral hypercalcemia of malignancy (HHM) (61, 62). It stimulates osteoclastic bone resorption and is present in all patients with HHM and in 75% of patients with breast carcinoma metastatic to bone, but is not detected in normal plasma (63). Studies utilizing the MDA-MB-231 intracardiac model indicate that PTHrP over-expression leads to enhanced bone disease and conversely, that reduced PTHrP expression inhibits bone metastases (60, 64). These data indicate that PTHrP may have an important role in metastatic disease (aside from hypercalcemia), as a mediator of bone destruction and has led to the proposal that it could be utilized as a predictive marker in primary tumors of subsequent bone

metastasis. This has been confirmed in some studies (65-67) but has not been confirmed in a large prospective study published recently (68). Here, it was shown that PTHrP is associated with a less invasive phenotype and that PTHrP expression in the primary tumor correlates with a reduced incidence of bone metastasis. The discrepancy between experimental studies using the MDA-MB-231 intracardiac model and the clinical data remains unexplained but reveals the importance of using clinically relevant models to study metastasis. Such a model is now available (see below), and has revealed that PTHrP expression in the primary tumor was not a predictor of bone metastasis (Tavaria et al., submitted for publication). Further, it was shown recently that whilst chemically induced mammary tumors in mice over-expressing PTHrP in their mammary glands developed with shorter latency than in wild type mice and metastasized to soft tissues, they did not metastasize to bone (69).

5. SYNGENEIC MODEL OF BREAST TO BONE METASTASIS

As stated above, the most useful model for studying the metastatic spread of breast cancer to bone is one that mimics all steps of the process in humans. This will include spontaneous metastasis from the mammary gland to distant sites, including bone, and a host with an intact immune system.

A model that fulfills these requirements now exists (70). Two cell lines called 4T1.2 and 4T1.13 have a high propensity to metastasize to bone after orthotopic injection into the mammary fat pad (70). They are derived from the 4T1 subline that was originally isolated by Miller and colleagues (71), as an extension of a study in 1978 by Dexter et al. (72) who isolated several distinct lines with differing metastatic capacities from a spontaneous BALB/cfC3H mammary tumor. The isolation of populations of cells with different metastatic properties from a single tumor confirms the heterogeneity of primary mammary tumors.

The 4T1.2 and 4T1.13 clones mimic the clinical disease in that a primary breast tumor grows following orthotopic inoculation into the mammary gland, the cells invade through the stroma into the circulation and colonize distant organs, including lymph nodes, lung and bone. Other sublines isolated by Miller et al. (71) are either non-metastatic (67NR) or weakly metastatic (4T07, 168FARN or 66c14) (Figure 1). Following mammary fat pad inoculation, bone metastases are evident within 25-30 days by histology (Figure 2) and hind leg paralysis is observed in some mice injected with the bone metastasizing clones. This is the first reported mouse model of

spontaneous metastasis of breast tumor cells from the primary site in the mammary gland to bone.

The designation of sites of metastasis is shown in Figure 1. These sites were confirmed by detection of overt metastatic nodules using histological techniques and by isolation and growth in culture of drug resistant tumor cells from various organs (70, 71). However, using a more sensitive method for detection of metastases, as described below, we have further refined the sites of metastasis for each of these sublines.

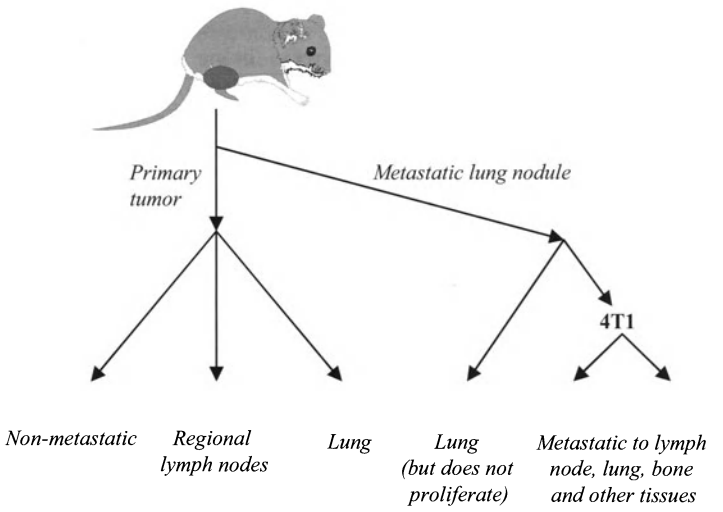

Figure 1. Orthotopic model of breast cancer metastasis to bone. Several tumor sublines have been isolated from a spontaneously arising mammary gland carcinoma. Each subline has a distinct metastatic phenotype. 67NR is non-metastatic, while 168FARN, 66cl4 and 4T07 are weakly metastatic and have a tissue restricted metastatic distribution. 4T1.2 and 4T1.13 are two bone metastasizing tumor clones derived from the lung metastasizing 4T1 subline.

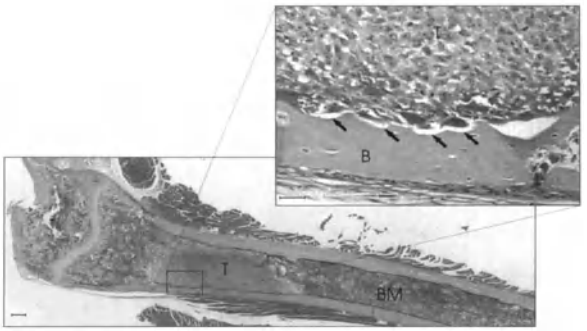

Figure 2. Hematoxylin and eosin staining of a section through the femur of a 4T1.2 tumor bearing mouse, 30 days after inoculation of tumor cells into the mammary gland (x20, scale bar 200mm). At higher magnification (x200, scale bar 25mm), resorption pits (arrows) can be observed in the bone (B) adjacent to the metastatic nodule (T). These pits are not evident in bone adjacent to normal bone marrow (BM).

5.1 Quantitative Measurement of Metastatic Burden

Each tumor line within the model has been tagged with a reporter gene to enable quantitative measurement of tumor burden in different organs by real time PCR. Reporter genes that we have used for this purpose include green fluorescent protein (EGFP), neomycin and puromycin resistance genes. EGFP was abandoned as a reporter gene after it was observed to cause reduced growth and metastasis in mice, presumably due to the EGFP protein acting as an antigen. A similar effect has not been observed with the other two reporter genes. In contrast, EGFP has proven to be a very useful imaging agent for metastases in xenograft models where immune rejection is not an issue (73). Tumor burden has been measured in multiple organs following inoculation of tumor cells into the mammary fat pads of mice (Eckhardt et al., manuscript in preparation). The analysis, reproduced in Figure 3, confirms and further expands the distribution of metastases detected histologically in mice, with the 67NR line failing to metastasize at all, and the other lines metastasizing to varying extents. This method of analysis enables detection of micrometastases not visible by histology. However, when the relative tumor burden increases, there is a good correlation between measurement of tumor burden using this method and by histological assessment (Tavaria et al., submitted for publication).

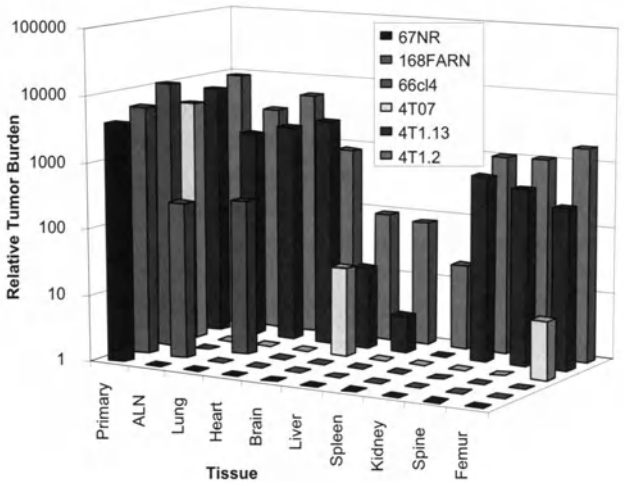

Figure 3. Detection of tumor burden in mouse tissues by real time PCR analysis. Tissue distribution of tumor cells tagged with the neomycin resistance gene following growth of a primary tumor in the mammary fat pad. The relative tumor burden is a measure of the ratio of neomycin positive tumor cells to total cells in the tissue (ALN, axillary lymph node).

5.2 Expression Analysis of the Sublines of the Model

The model is of enormous value for a comparison of the expression profiles of primary tumors with different metastatic capacities, since all sublines are derived from the same spontaneous mammary tumor and are genetically well matched. By cDNA microarray analysis, a number of candidate genes that may have a role in specific metastasis to bone and/or other sites have been found. Using small primary tumors with no necrotic regions (5-7 mm diameter), we have examined the profile of expressed genes using RNA isolated from each tumor. Competitive hybridization to the NIA 15k cDNA array of each metastatic line compared to the non-metastatic 67NR line has generated a number of genes potentially important in metastasis of breast cancer (Eckhardt et al., submitted for publication).

These genes include caveolin-1, which is a regulator of signaling pathways that function through caveolae (74). Loss of caveolin-1 is associated with enhanced metastatic capacity in the murine model and poorer survival in breast cancer patients (Sloan et al., manuscript in preparation). The integrin β3 is another candidate gene for bone metastasis. Increased integrin β3 expression is associated with increased metastasis and enforced expression of this integrin in a poorly metastatic line enhances

metastasis, despite causing a reduced growth rate of the primary tumor (Sloan et al., manuscript in preparation). Other interesting candidate genes include protease nexin-1 (a serpin), urokinase plasminogen activator (uPA), a newly described integrin binding protein called nephronectin (75) and a number of ESTs.

5.3 Induced Expression of Genes by Stromal Cells within the Tumor

Whilst the genes mentioned above are differentially expressed in primary tumors with differing metastatic patterns, it is important to recognize that some may be expressed by stromal cells within the tumor rather than by tumor cells. It is now well established that tumor cells exert an influence on the behavior of surrounding normal cells, including vascular endothelial cells, fibroblasts, and cells of the immune system (3, 76). The contribution of these other cell types, including their interaction with the epithelium, requires investigations into the expression profiles of specific cell types. Using this model, specific cell populations from fresh primary tumors of varying metastatic potential and matched metastatic tumors can be immunopurified and analysed independently. Alternatively, laser capture microdissection (LCM) can be used to isolate tumor and stromal cells from frozen tissue. These techniques will potentially be more useful in finding genes important in the stroma and blood vessels, which may be masked when analysing whole tumor preparations. This will also be required in the bone environment to identify the key players in bone resorption, and whether the stroma initiates the tumor production of genes such as PTHrP.

5.4 Therapeutic Applications of the Model

Since the highly metastatic 4T1.2 and 4T1.13 sublines closely mimic aggressive human breast cancer, they allow us to examine the importance of genes found by cDNA microarray analysis in the metastatic process. This can be achieved with relative ease (depending on the gene under study) by using either antisense expression or by stable RNA interference. In addition, induced expression of potential metastasis genes in the less metastatic lines such as 66cl4, may reveal a role for that gene in enhancing metastasis. The mechanism by which each gene functions to promote or suppress metastasis and its potential as a therapeutic target can be determined. The relevance of both novel genes and those previously implicated in the literature, such as PTHrP, can be tested in this model. The quantitative tumor burden assay enables accurate analysis of the impact of the particular gene in metastasis.

It is important to verify that the gene under analysis is relevant to human breast cancer metastasis as well. The development of human tumor tissue arrays has enabled us and other investigators to link expression of a particular gene to clinical outcome for that patient, using a large number of samples arrayed on one slide. If such a correlation is found, the gene becomes a potential target for drug therapy or a useful prognostic marker.

Novel compounds or drugs for which there is some evidence of inhibition of metastasis or tumor growth in bone can be tested in this model with relative ease, again using the relative tumor burden assay as a quantitative measure of drug efficacy.

The development of improved models of breast cancer metastasis to bone and new technologies such as cDNA microarrays and tissue arrays are combining to enable an accurate molecular analysis of this debilitating disease. New molecular targets and specific therapies will be the likely outcomes of these studies.

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Chapter 2

QUANTITATION OF BONE METASTASIS IN EXPERIMENTAL SYSTEMS

Julie A. Sharp[1] and Erik W. Thompson[1, 2,]
[1]*Department of Surgery, University of Melbourne, St. Vincent's Institute of Medical Research, and* [2]*Bernard O'Brien Institute of Microsurgery, St. Vincent's Hospital, Fitzroy, Australia*

1. INTRODUCTION

Animal models are important tools to investigate the pathogenesis and develop treatment strategies for bone metastases in humans. However, there are few spontaneous models of bone metastasis despite the fact that rodents (rats and mice) and other animals (dogs and cats) often spontaneously develop cancer. Therefore, most experimental models of bone metastasis in rodents require injection or implantation of neoplastic cells into orthotopic locations, bones, or the left ventricle of the heart.

2. METASTATIC BONE DISEASE

Bone is a frequent site of metastases of the most common tumors, e.g., breast carcinoma and prostate carcinoma. The functions of the skeleton, calcium homeostasis and mechanical support, are carried out by the continuous destruction and rebuilding of bone, a process known as bone remodeling. Multinucleated, hemopoietically derived osteoclasts, which are related to macrophages, digest the bone, and mesenchymal-derived osteoblasts rebuild it. This is kept in balance by finely regulated processes whereby osteoblast lineage cells respond to homeostatic signals and release factors that regulate osteoclast generation and activity. Cells that participate in inflammation and immunity also can stimulate osteoclast formation and

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lead to bone destruction. Tumor cells most likely interfere with these physiologic processes, seeding within the bone and establishing metastases. Bone metastases from breast carcinoma are generally associated with bone destruction which occurs in response to factors secreted by metastatic cells. Some of these factors secreted by the metastatic cells activate osteoclasts, although in some cases others are proteases that degrade bone collagen. In contrast, the balance is altered in prostate carcinoma bone metastases, which are predominantly osteosclerotic.

2.1 Animal Models

Injection of cancer cell lines into the left cardiac ventricle of immunodeficient mice is a widely used animal model of bone/bone marrow metastasis. This experimental setting closely resembles the clinical situation of minimal residual disease after removal of the primary tumor. However, detection of bone metastasis by radiography makes it a less-than-ideal model of minimal residual disease. In fact, radiologically evident osteolytic/osteosclerotic metastases are a late and macroscopic event in the evolution of metastatic bone disease. Thus, the model lacks the sensitivity that would be necessary to dissect the initial processes, such as the "angiogenic switch," essential for tumor progression. Furthermore, the radiological detection of osteolytic and/or osteosclerotic metastases is an indirect measure of the tumor burden. Only a parallel histomorphometric analysis allows the distinction between a therapeutic effect exerted directly on tumor cells or indirectly through inhibition of bone resorption. Therefore, more sensitive methods to detect and monitor directly metastatic growth in bone marrow/bone of whole animals need to be developed.

2.1.1 The MDA-MB-231 Breast Cancer Model

Since bone metastasis occurs very rarely from current spontaneous or experimental metastasis models of carcinoma cells in immunocompromised mice, an arterial seeding model involving the direct intracardiac injection of the cells into the left ventricle has been developed to mimic the process of metastasis (1, 2). This model has also been used for orthotopic injection into the mammary fat pad and tibia. The breast cancer cell line MDA-MB-231 has been widely used as a working model for *in vivo* metastatic studies in immunocompromised mice. MDA-MB-231 cells have a high propensity to metastasize to bone and soft organs when injected intracardially, grow well as xenographs, metastasize to the lymph nodes and lungs from the mammary fat pad, and grow well within the bone environment of the tibia. For these

reasons this model offers a great medium to study many aspects of cancer metastasis and treatment.

3. QUANTITATION IS A KEY ISSUE

3.1 Bone Damage

Bone destruction can be monitored by X-ray, mammography, Faxitron or phase contrast imaging, all which allows visualization of the degree of bone degradation caused by the metastatic tumor. Histomorphometry techniques can be used to provide more information about tumor/bone interactions and impact the tumor has on the bone environment.

In some cases, metastases can be osteoblastic which is a result of factors secreted by the tumor cells influencing the osteoblasts and results in increased bone density and bone deposits. These types of lesions can also be monitored by these techniques.

3.1.1 Histomorphometry and Animal Models of Metastasis

Bone histomorphometry provides quantitative information about bone mass, architecture and turnover. Undecalcified sections are prepared using methyl methacrylate embedding and decalcified sections with paraffin or glycol methacrylate. A variety of histochemical and immunohistochemical stains can be performed to measure cellular counts of osetoblasts and osteoclasts, mineralizing surface, mineral absorption rate, bone formation rate, bone porosity, bone volume, osteoid surface (volume and thickness) and proliferation rates of tumor cells.

In experimental models of bone metastasis, there is evidence that osteolysis and colonisation of the bone marrow by the tumor can be reduced by strategies that inhibit the release or production of proteases (3, 4) or by overexpressing tissue inhibitors of matrix metalloproteinases in metastatic cells (2). The matrix metalloproteinases are mediators of homeostatic bone growth and remodeling (5) and are likely to contribute to the invasion and metastasis of malignant tumors in bone (6, 7). On this basis, Lee et al. (8) have postulated and shown that a synthetic inhibitor of matrix metalloproteinases, Batimastat (BB-94), inhibits the activity of matrix metalloproteinases expressed by MDA-MB-231 human breast carcinoma cells, and blocks the ability of these cells to degrade osteoblast-like matrices or to form resorption pits in cortical bone. *In vivo*, where more than one cell type may be involved in bone destruction, treatment of tumor-bearing

animals with Batimastat inhibited tumor-associated osteolysis, tumor growth, and the replacement of marrow by tumor. By using histomorphometry to analyse the metastatic lesions, the ability to distinguish between osteolysis, tumor burden, and marrow replacement was achieved which can not be accomplished by more conventional radiological measurements. The effects of Batimastat treatment on the formation of bone metastases by MDA-MB-231 cells were striking. The marked osteolytic effects of the tumor were blocked in both femoral and vertebral bone, accompanied by diminished extension of tumor into the medullary long bone, decreased tumor volume, sparing of haematopoietic tissue, and a decrease in the number of metastases found in the vertebral bodies. Winding et al. (9) also found considerable inhibition of bone metastasis in the MDA-MB-231-B (bone-metastasis selected) model using either Batimastat or Galardin (GM-6001, another broad-spectrum MMP-inhibitor), and in fact were able to reverse established bone damage. These lesions were also assessed radiologically with morphometric analysis. Our own group has found similar retardation of the onset of osteolytic metastasis monitored with weekly Faxitron, using the gelatinase-selective MMP inhibitor Prinomastat [AG 3340; Agouron-Pfizer, (10)].

3.2 Tumor Burden

Quantitation of tumor burden has been demonstrated in numerous ways using essentially the same technique. Quantitative real time PCR provides an accurate and sensitive modality for detection of tumor cells by amplifications of a given sequence that is unique to the cells employed. These sequences include the bacterially derived B-galactosidase (Lac-Z) and neomycin resistance genes, the luciferase gene and the gene encoding green fluorescent protein (GFP), all which have been genetically introduced into cancer cell lines to identify and quantitate the metastatic cells within the host rodent model organism. Another method recently being used employs the detection of human specific Alu sequences that are a normal component of the human genome to identify and quantitate the metastatic human cells within the host organism.

3.2.1 Quantitation of *in vivo* Breast Cancer Models

We employ the human breast cancer cell line MDA-MB-231 transduced with a bacterially derived Lac Z sequence (MDA-MB-231-BAG) as a model of breast cancer metastasis. A sensitive quantitative real-time polymerase chain reaction (PCR) method that amplifies the genomically incorporated Lac Z sequence in the MDA-MB-231-BAG cells is used to detect and

quantitate bone and soft organ metastasis in nude mice intracardially inoculated with these cells. Amplification of this sequence enables us to specifically detect these cells in mouse organs and bones. PCR detection was linear, specific, more sensitive than conventional PCR, and could be used to directly quantitate metastatic burden in bone and soft organs. Attesting to the sensitivity and specificity of the PCR detection strategy, as few as several hundred metastatic MDA-MB-231 cells were detectable in 100 micron segments of paraffin-embedded lung tissue, and only in samples adjacent to sections that scored positive by histological detection (Figure 1). Moreover, the measured real-time PCR metastatic burden in the bone environment displayed a high correlation to the degree of osteolytic damage observed by high resolution X-ray analysis (Figure 2). Such a direct linear relationship to tumor burden and bone damage substantiates the so-called 'vicious cycle' hypothesis in which metastatic tumor cells promote the release of factors from the bone which continue to stimulate the tumor cells. The model provides a useful tool for molecular and cellular analysis of human breast cancer metastasis to bone and soft organs, can easily be extended to other cell /marker /organ systems, and should also find application in preclinical assessment of anti-metastatic modalities (11).

A similar model has also been used for detection of a murine mammary adenocarcinoma cell line (Clone 66) transduced with neomycin resistance gene injected orthotopically into immunocompromised mice for detection of cells within bone marrow. PCR for the neomycin gene performed on bone marrow cells harvested from tumor bearing mice demonstrated as few as 10^2 injected tumor cells produced bone marrow micrometastases at 4 weeks postinjection. Small foci of tumor cells were identified in the mammary fatpad without gross evidence of primary tumors. Higher doses of tumor cells produced bone marrow micrometastases, detectable by PCR, at one week post-injection. Constructs containing GFP and the neomycin resistance gene were also transduced into Clone 66 cells (Cl66-GFPneo) and injected into the mammary fat pad. GFP transduced tumor cells were identified in multiple tissues in addition to bone marrow by flow cytometric analysis (FACS) but less than 13% of the animals developed gross metastases. This model is a clinically relevant tool for the analysis of organ specificity of metastasis (12).

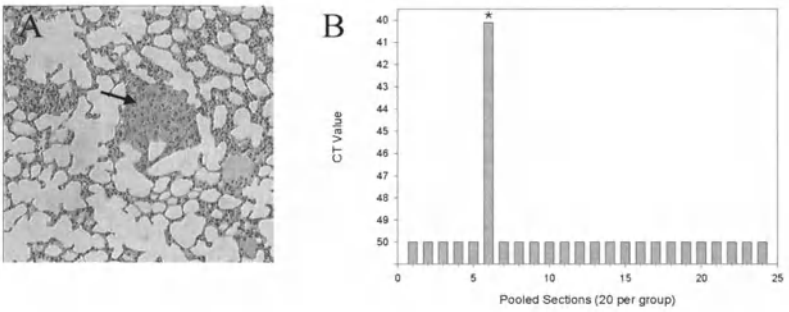

Figure 1. Analysis of micrometastases in mice lungs by real-time PCR and histology. Animals were inoculated with MDA-MB-231 BAG cells into the mammary fat pad. After 45-55 days the mice were sacrificed, the lungs removed, and any micrometastases analyzed by histology and real-time PCR. A) Haematoxlyin and eosin stained section of mice lung tissue showing micrometastasis (arrowed). B) Real-time PCR of DNA extracted from a series of paraffin embedded sections across the entire lung. Each group represents 20 pooled sections within the series. The asterisk corresponds to the group of sections represented by A.

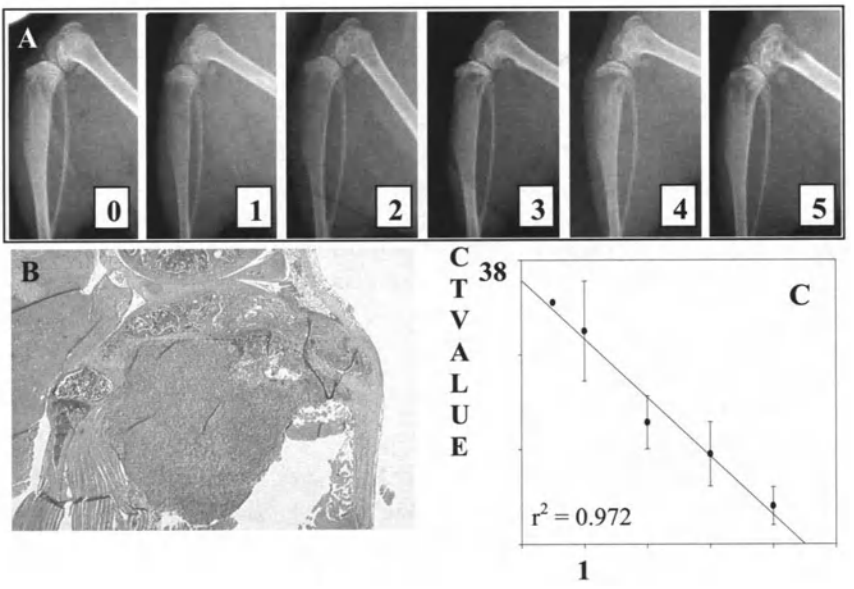

Figure 2. Comparison of the metastatic burden in bone and the degree of osteolytic damage observed. The long bones from mice following intracardiac inoculation of MDA-MB-231 BAG cells were analyzed by high resolution X-ray analysis and by real-time PCR. The degree of osteolytic damage was scored on a semi-quantitative scale, with a score of 0 representing no visible damage and a score of 5 representing severe damage. Examples are illustrated in panel (A), and a haematoxlyin and eosin stained section of tumor growth in the tibia region for a damage 'rating 4' is given in panel (B). There was a direct correlation between the severity of osteolytic damage observed and the metastatic burden in bone detected by real-time PCR (expressed as CT value) panel (C). The error bars represent the range of the number of cancer cells detected within the different damage scales. This figure is reprinted from Clinical Experimetal Metastasis Vol. 19(5): 377-383, Figure 5, Copyright 2002 with kind permission of Kluwer Academic Publishers.

3.2.2 Pain

Bone cancer pain is very common, and this type of pain can be extremely debilitating. Development of an experimental model for studying this condition is critical to advancing an understanding of the mechanisms that cause pain in patients with malignant disease.

A murine model of bone cancer has been used to combine the extent of tumor-induced bone destruction, pain, and neurochemical characterization of the peripheral and central nervous systems in order to investigate bone cancer pain. Disease-induced bone destruction was assessed by radiographs and histomorphometry. Pain was assessed by spontaneous and elicited

behaviors, and neurochemical analysis involved immunohistochemical detection of hyperalgesic peptides and neurochemical markers.

Mice with distal femoral sarcomas exhibited behavioral and neurochemical measures of pain. The pain condition created by malignant bone disease was distinct neurochemically from inflammatory and neuropathic pain states. Experimental evidence indicated that both disease-induced osteolysis and tumors themselves contributed to the generation of pain and that peripheral and central sensitization of the nervous system was present (13). Interestingly, one of the major benefits of bisphosphonates, which retard osteolytic destruction of bone and are used clinically in breast and prostate cancer, is pain reduction.

3.2.3 Real Time Imaging

The development of new anticancer strategies requires more sensitive and less invasive methods to detect and monitor *in vivo* minimal residual disease in cancer models. Improvements in the imaging of experimental animals *in vivo* using fluorescent markers or light emitted from luciferase have led to increased sensitivity of detection and more accurate quantification of bone metastases which can be detected externally by sensitive detection systems. Cancer cell lines permanently transfected either with the firefly luciferase (*Luc*) or GFP have been used to monitor local tumor growth (14) and development of metastasis to different organs in living mice (15). Clinical and experimental observations indicate that the hematopoietic marrow, rather than the bone tissue, is the initial site of cancer cell seeding. Indeed, small clusters of cancer cells (micrometastases) can be detected in a vast proportion of the bone marrow aspirates of patients affected by a variety of epithelial cancers with no radiological or scintigraphic evidence of bone metastasis at the time of diagnosis and/or surgery of the primary tumor (16). Micrometastases represent the pathophysiological basis of minimal residual disease (17-19) that will eventually lead to cancer relapse as overt metastases. However, they cannot be detected by conventional staging methods and are poorly influenced by current treatment (19). Hence, there is a need for alternative therapeutic strategies that should be tested in experimental models able to mimic micrometastatic spread.

For example, imaging of human prostate carcinoma PC-3M cells transfected with luciferase, following injection into the left ventricle, has demonstrated that there is rapid localization of tumor cells to bones and other organs, such as the kidneys and lungs. Wetterwald et al. (20) have demonstrated that whole-body bioluminescent reporter imaging (BRI) effectively allows sensitive localization and growth monitoring of minimal

metastatic deposits in the bone marrow at a stage largely preceding tumor-induced osteolysis.

3.3 Prostate Cancer Models

Prostate cancer metastasis has two defining clinical characteristics: a tendency to involve bone and a marked osteosclerotic response of the bone to the presence of metastatic cells. Prostate cancer bone metastasis causes tremendous morbidity, including pain, impaired mobility, pathologic fractures, spinal cord compression, and other problems. Gross, clinically significant, metastatic deposits develop in bone long before metastases to soft viscera become apparent (21-23). The marked increase in the turnover of bone matrix associated with the presence of prostate cancer cells in bone is often recognized as an osteosclerotic response on radiographic imaging studies. It is well accepted, however, that both bone formation and bone breakdown are present within metastatic deposits (24-26).

Nemeth et al. (27) described the SCID (severe combined immunodeficient)–human model of prostate cancer metastasis, in which a variety of human and mouse organ environments were implanted into SCID mice to serve as a target for human prostate cancer cells. They found that a variety of human prostate cancer cell types preferred to form tumors in the human bone environment than in other human or mouse organ environments. They also demonstrated a bone response ranging from mostly osteolytic to mostly osteoblastic, depending on the type of prostate cancer cells introduced into the bone. Bone tumors formed by the PC3 prostate cancer cell line were consistently osteolytic in nature, suggesting that this model could be used to study the degradative portion of the bone turnover cycle. Tumors developed in the human bone cavity but not in the mouse skeleton. A similar system involving non-obese diabetic (NOD)/SCID mice implanted with adult human bone fragments has also been described recently (28). While these models can be used to explore prostate cancer growth potential in bone, they are not useful for exploring the metastatic events that result in tumor cells establishing in the bone environment. Tumor cells can colonize the human bone fragment after either intravenous injection or direct injection into the bone fragment but not after growth of a primary tumor. The models do, however, avoid the incompatibility issue of human tumor cells in murine stromal tissue. A final point that distinguishes the few existing prostate cancer models from the real disease is the fact that PC3 and DU-145 tumors are osteolytic, whereas those of LnCaP are both osteolytic and osteoblastic (27). The metastases of human prostate cancer are predominantly osteoblastic or mixed blastic and lytic.

Most research into human prostate cancer uses just three cell lines, PC3, DU-145, and LnCaP. After subcutaneous inoculation into immunocompromised mice, these lines grow as tumors but do not metastasize. Orthotopic inoculation can result in some limited metastasis, but still there is the unresolved issue of human tumor tissue in a murine host. The mouse prostate reconstitution model is a step forward, in that normal prostate epithelial cells are transformed with myc and/or ras, mixed with normal mesenchymal cells, and inoculated into the renal capsule of recipient mice (29). These mice develop metastatic lesions in the lung, liver, mesentery, and bone. Elegant as the model is, however, this is an artificial transformation. Like all other models where single or multiple oncogenes are overexpressed, the events do not necessarily mimic the complex processes leading to prostate or other cancers.

3.4 Transgenic Models

New transgenic mouse models of cancer have a low incidence of spontaneous bone metastasis, but cell lines derived from these tumors can be selected *in vivo* for increased incidence of bone metastasis. It is essential to validate and correctly interpret the lesions in models of bone metastasis to accurately correlate the data from animal models to human disease. Animal models have provided support for the "seed and soil" hypothesis of bone metastasis.

4. CONCLUSION

Current and future animal models will continue to be important tools to investigate the pathogenesis and treatment of bone metastases in humans. Animal models of metastasis have supported drug development and have been useful for identification of metastasis suppressor and promoter genes as novel targets for the development of novel therapies. Further refinement of these models will involve analysis of the metastatic process by imaging and use of image data to stage disease and guide tissue sampling for gene expression profiling via gene array technology. In the future, integrated analyses of these models will be needed to understand the complexities of this important disease process (30).

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Chapter 3

COMPARATIVE MORPHOMETRIC STUDY ON BONE REMODELING IN HUMAN SPECIMENS AND IN EXPERIMENTAL MODELS OF METASTATIC BONE DISEASE

Snezana Vukmirovic-Popovic, Eric Seidlitz, F. William Orr*, and Gurmit Singh
Juravinski Cancer Centre, Mc Master University, Hamilton Ontario, Canada
**Department of Pathology, University of Manitoba, Winnipeg, Manitoba, Canada*

Metastatic cancer is the most common malignant tumor affecting bone and is accompanied by significant clinical morbidity, including pain, osteolysis, pathological fracture, spinal compression syndromes and hypercalcemia. Bone destruction is a marked clinical feature and the major source of morbidity associated with bone metastases. Most of these clinical features can be related to structural changes in bone that are caused by effects of the tumor on normal processes of bone remodeling.

1. QUALITATIVE HISTOLOGICAL OBSERVATIONS IN CLINICAL SAMPLES

During physiological bone remodeling, bone resorption by osteoclasts is closely coupled to bone formation by osteoblasts to maintain dynamic homeostasis. Once cancer colonizes bone, tumors can cause two distinct but overlapping types of skeletal lesions (i.e., osteolytic and osteoblastic) (1, 2, 3). In malignant osteolysis, a response often seen in breast cancer, the coupling process is dysregulated showing dramatic increases in bone resorption, impaired secondary osteoblast responses, and a net bone deficit

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(1, 4, 5). In prostate cancer, the presence of tumor cells in the bone often changes osteoblastic activity, resulting in reduced formation of normal lamellar bone, increased formation of abnormal woven bone, and net bone overproduction (6). To some extent there is almost always bone formation on a microscopic level in osteolytic bone metastasis, even if it can not be detected by standard imaging modalities (1). There are quantitative differences in osteoclastic bone resorption between breast carcinoma and the other types of metastatic carcinoma, such as renal and sqamous cell carcinoma (7, 8).

It is generally accepted that trabecular bone strength depends not only on bone volume but also on its structure (9, 10). Bone fractures are one of the most important complications and a main source of morbidity in patients with bone metastasis. Breast cancer is the most common cause of pathological fractures. (5, 11). Because malignancy-associated focal bone loss at sites of tumor osteolysis produces different clinical manifestations (e.g., fracture/no fracture), it is likely that the pathophysiologies of these processes differ. For example, our previous comparison of patients with and without pathological fractures revealed differences in bone remodeling judged by histomorphometry and microstructural examination. Furthermore, node-strut analysis showed increased disconnectivity and decreased connectivity suggesting more pronounced microstructural changes in the fracture group compared to the non-fracture (12) group. Bone fracture can also be a manifestation of metastatic prostate carcinoma (13). Reduced formation of normal lamellar bone and production of woven bone which is less strong than lamellar bone might contribute to bone strength and fracture risk.

1.1 Histological Patterns in Human Bone Biopsies

The most commonly encountered microscopic bone reaction in metastatic breast cancer is a mixed picture of both osteolysis and osteosclerosis (1). Our study on bone biopsies from patients with metastatic breast carcinoma revealed varying degrees of morphological changes related to bone resorption and bone formation. Osteoclast numbers, lacunar osteolysis, and trabecular fragmentation varied from case to case. Bone trabeculae were surrounded by sheets of malignant cells with tubule formations or solid cell clusters, with varying amounts of fibrovascular stroma. Morphological findings of bone formation included different presentations such as onion-like sclerosis layers on inactive bone surface, spongy sclerosis on active bone surfaces and networks of primitive woven bone in fibrovascular stroma (Figure 1a, b). Different histological findings were observed in the occult metastasis compared to the symptomatic bone metastasis with radiologically

detectable bone lesions. Although, histologically a large part of the bone marrow was replaced by metastatic tumor, extensive osteolysis or osteoclastic bone resorption was not observed. There was also evidence of new bone formation rising from the quiescent bone surfaces (Figure 1c, d).

Osteosclerosis is the next most common predominating bone reaction, often seen in prostate cancer and less common in lung cancer (1). Prostate cancer metastases are predominantly osteosclerotic, but woven bone formation is accompanied by progressive lamellar bone destruction. Areas of erosions are also seen on the surface of bony trabeculae in regions free of tumor. The analysis of human bone specimens in polarized light reveals osteoclastic bone resorption of lamellar bone replaced by new abnormal woven bone producing the well-recognized bone forming metastasis (14).

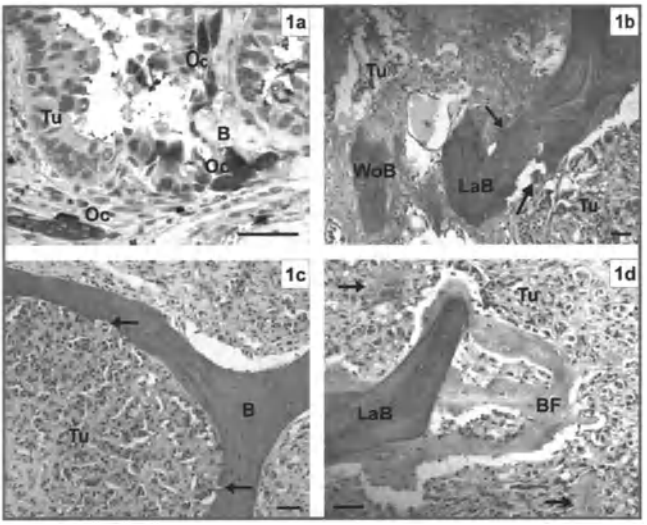

Figure 1. Representative histological findings in bone metastases. Femoral bone biopsies from patient with metastatic breast carcinoma.

(a) Osteolytic bone metastasis. TRAP-positive osteoclasts on the bone surface and in the tumour tissue surrounding the bone trabecula (original magnification x400; TRAP staining).

(b) Reactive new bone formation on one side of a lamellar bone trabecula (thin arrow), with osteoclast-mediated bone resorption on the opposite side of the trabecula (thick arrow). New stromal woven bone formation with irregular orientation of collagen fibers in contrast to the regular orientation in lamellar bone (original magnification x100; H&E staining, polarized light).

(c) Occult bone metastasis with rarely observed microresorptions (arrows) along bone trabeculae (original magnification x400, H&E staining).

(d) Occult bone metastasis showing de novo bone formation on quiescent bone surfaces and in tumour stroma (arrows) (original magnification x400, H&E staining, polarized light).

The rarely reported intertrabecular type of metastasis is characterized by an infiltration of tumor cells into the bone marrow space without a significant alteration of the trabecular bone and no visible lesions by radiology (2, 15, 16). Radiologically undetectable metastatic lesions have been documented as "occult bone metastasis" (17). Some oncologists differentiate bone metastasis with bone destruction from bone marrow metastasis with no bone destruction (18). The mechanism underlying the development and changes in bone remodeling of this type of metastases has not been extensively examined. Based on our recent study using bone histomorphometry and node-strut analysis, occult metastasis represents a separate subtype of metastasis compared to the symptomatic bone metastasis (12).

2. EXPERIMENTAL MODELS OF BONE METASTASIS

Several tumor models have been used to study bone metastasis experimentally. Intracardiac injection has been widely employed as a means to obtain bone colonization by established non-human tumor cell lines in immunocompetent animals or human cancer cell lines in immunodeficient animals (19, 20, 21). Animal models of bone metastasis can provide excellent information about tumor-bone interactions and the mechanisms of action of tumor cells during bone colonization at the tissue level. For example, the estrogen-independent human breast cancer cell line, MDA-MB-231 cells is widely accepted as a suitable model to study osteolytic bone metastasis (22, 23). Unfortunately, osteosclerotic type of prostate cancer metastasis to bone has been difficult to mimic in animal models. Osteolysis is the predominant metastatic phenotype in animals injected with prostate cancer cells (e.g., PC-3, LNCap, PA III) (20, 24, 25).

The syngeneic Dunning MAT-LyLu rate prostate cancer model has been argued to be the first described primarily osteoblastic model (26). Human, estrogen dependent breast carcinoma cells, MCF-7 have also been reported to develop osteosclerotic bone metastases after initial bone resorption (21). However, the bone formation capacity of estrogen-dependant human breast cancer cells (e.g., MCF-7 and T-47D) is still not clear since, in order to support the growth of these breast cancer cells, it is necessary to give supplementation of estrogen because there are insufficient levels of estrogen in female nude mice. It is possible that the administration of estrogen in this model could stimulate bone formation, leading to misinterpretation of the model as osteoblastic bone metastatic model (20).

2.1 Qualitative Histological Observations

Using previously described procedures (19, 26, 27, 28), we have studied the morphological and morphometric alterations of bone remodeling in three animal models of bone metastases. First, the MDA-MB-231 tumor in female nude mice (BALB/c nu/nu) has been found to be a hypercellular tumor that causes an osteoclastic bone resorbing response four weeks after cell injection. A stromal host bone response, commonly seen in human bone metastasis, was not present (Figure 2a, b, c). Second, the murine melanoma B16/F1 tumor in C57BL/6 female mice has been found to be a very aggressive tumor with extensive tumor necrosis and massive bone cell colonization 12 days after cell injection, forming osteolytic bone metastases (Figure 2). The Mat-LyLu tumor, forming bone metastases approximately 12 days after cell inoculation in male Copenhagen rats, causes osteolytic femoral bone metastases, associated with extensive tumor necrosis and osteoclastic-mediated bone resorption. However, spinal bone metastases in this tumor were of a mixed osteoblastic-osteolytic type. Stromal new bone formation as a result of intramembranous ossification was associated with development of a fibrous stroma in the host bone (Figure 3). Reactive new woven bone formation, arising from quiescent bone surfaces, was also noted. Subperiosteal colonization was present in both MDA-MB-231 and MAT-LyLu *in vivo* models (Figure 2c, f). Subperiosteal tumor metastasis is a typical metastatic site in animal experimental models that is not often present in human bone metastasis. It has been previously explained by tumor cell colonization based on anatomical arterial microvascularization of animal bone (20). Subperiosteal tumor localization can produce extensive cortical bone metastasis leading to cortical perforation. Bone metastasis from lung cancer is the only described human periosteal metastasis (20). Human skeletal metastases are most often intramedullary and typically involve the cortex only in the advanced phase of metastatic bone disease (7).

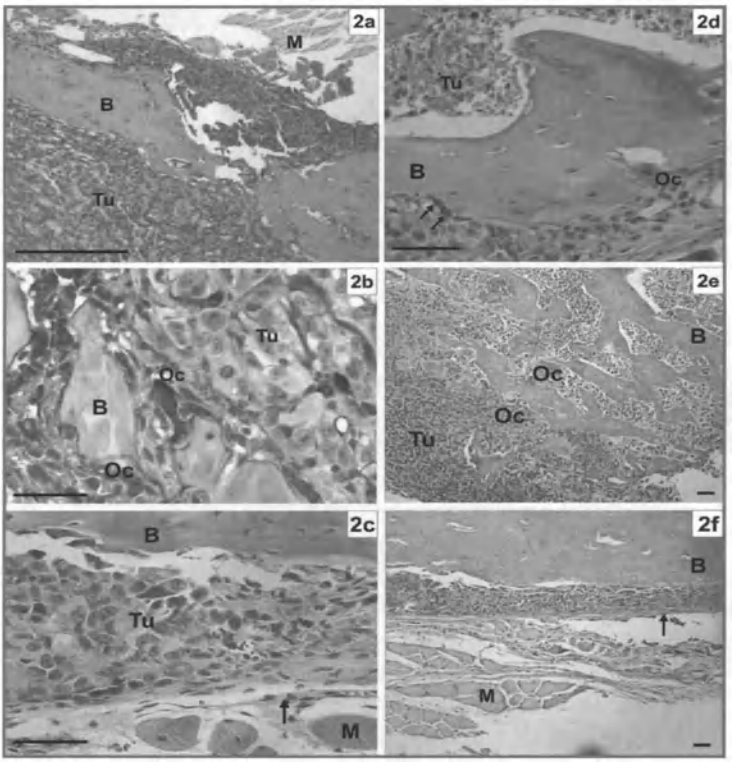

Figure 2. Animal models of osteolytic femoral bone metastasis.

(a) MDA-MB-231 breast cancer metastasis with extensive marrow colonization and cortical perforation invading into the surrounding muscle tissue (original magnification x400, H&E staining).

(b) MDA-MB-231 breast cancer metastasis with numerous TRAP-positive osteoclasts on the bone surface and in the tumour tissue (original magnification x400; TRAP staining).

(c) Subperiosteal MDA-MB-231 cell colonization involving the space between the cortex and periosteum (arrow) (original magnification x400; H&E staining).

(d) B16/F1 melanoma osteolytic femoral bone metastasis with extensive zones of tumour necrosis, numerous pigmented tumour cells on bone surface (arrows) and osteoclastic bone resorption (original magnification x400, H&E staining).

(e) MAT-LyLu osteolytic femoral bone metastasis with osteoclastic bone resorption and extensive tumour necrosis (original magnification x400, H&E staining).

(f) Subperiosteal MAT-LyLu cell colonization involving the space between the cortex and periosteum (arrow) (original magnification x100, H&E staining).

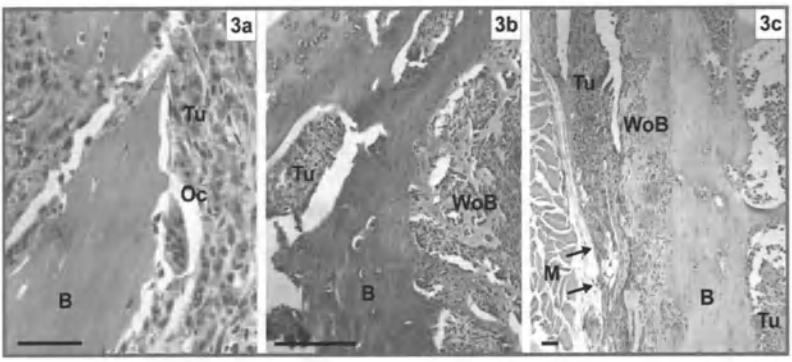

Figure 3. MAT-LyLu mixed type of spinal bone metastasis showing osteolysis and osteosclerosis.

(a) Osteoclastic bone resorption (original magnification x400, H&E staining).
(b) Reactive new woven bone formation (original magnification x400, H&E staining).
(c) Subperiosteal new woven bone formation (original magnification x100, H&E staining, polarized light).

A comparative example of breast carcinoma from clinical samples and in a corresponding animal model is shown in Figure 4. Human breast carcinoma often consisted of abundant fibrous stroma in metastatic bone lesions. Stromal new bone formation as a result of intramembranous ossification was associated with development of a fibrous stroma (Figure 4a, b). The MDA-MB-231 tumor was hypercellular, lacking a desmoplastic reaction or new bone formation. We noted that MDA-MB-231 tumor occasionally formed gland-like structures morphologically resembling human metastatic breast carcinoma. It may be that the bone microenvironment in the MDA-MB-231 tumor model has an important role in cell differentiation. This notion was also supported by various morphological tumor appearances in the soft tissue. Tumor cells in the soft tissue (e.g., muscle) were undifferentiated, resembling cells in tissue culture (Figure 4c, d).

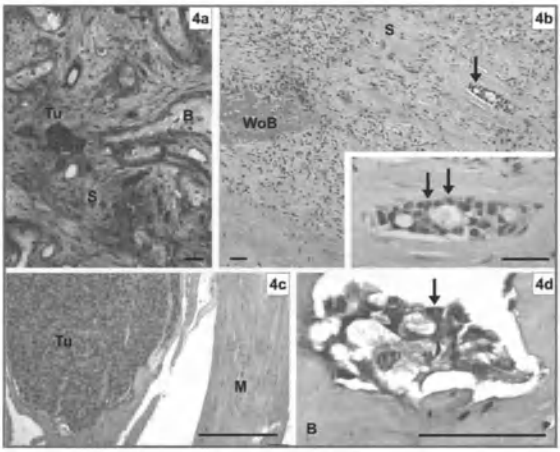

Figure 4. Comparative histological findings of bone metastasis in human specimens and experimental animal models.

(a) Undecalcified plastic-embedded human bone sample from a patient with metastatic breast carcinoma. Tumor cells and marked fibroblastic stroma surrounding the bone trabeculae (original magnification x100, Sanderson's TM Rapid Bone Stain, Van Gieson counterstain).

(b) Human specimen of metastatic breast carcinoma. Stromal new woven bone formation with gland-forming tumor nest of breast carcinoma (arrow) (original magnification x100, H&E staining). Insert- A well-preserved cribriform gland pattern of breast carcinoma (arrows) surrounded by abundant fibroblastic stroma (original magnification x400, H&E staining).

(c) MDA-MB-231 tumor. Undifferentiated tumor in the muscle compared to the tumor differentiation in bone environment (original magnification x400, H&E staining).

(d) MDA-MB-231 tumor. Gland forming metastatic tumor nest resembling human breast carcinoma (original magnification x400, H&E staining).

B-bone, BF-bone formation, LaB-lamellar bone, M-muscle, Oc-osteoclast, S-stroma, Tu-tumor, WoB-woven bone. Bar-50μm.

3. MORPHOMETRIC ALTERATIONS IN BONE METASTASIS

The numbers of quantitative morphometric studies of metastatic bone disease on human bone biopsies are still limited, especially when considering the importance of the effects of metastasis on bone loss and on bone strength. Although data from experimental animal models (19, 20, 21, 23) have provided general information about the effect of metastasis on the mechanisms of bone remodeling, the effect of tumor in humans can only be

studied in human bone. Well-documented comparative reports on the morphometric changes in animal models are also relatively scarce.

3.1 Bone Histomorphometry and Node-Strut Analysis

We have previously described a bone histomorphometric method performed on bone samples obtained from patients with metastatic breast carcinoma and from animal models (12, 28). To reduce intrabone variation, we evaluated at least two sections throughout the biopsy at 100-μm intervals, at least ten microscopic fields per sections (x100 magnification).

The nomenclature and symbols used in conventional bone histomorphometry were expressed according to Parfitt et al. (29). The following parameters were measured: eroded surface (ES/BS), osteoclast surface (OcS/BS), osteoclast number per bone surface (N.Oc/BS), osteoid volume (OV/BV), osteoid surface (OS/BS), osteoblast number per bone surface (N.Ob/BS) and cancellous bone volume (BV/TV). Trabecular thickness (Tb.Th) was calculated as the BV/TV divided by half the bone surface (BS) (30, 31). Parameter related to tumor include tumor volume (TuV/TV), expressed as a fraction of total tissue volume.

Two-dimensional parameters of trabecular structure were measured by node-strut analysis according to the methods of Mellish et al. (32). The microanatomical structural parameters as indices of bone structure included: node number (N.Nd), terminus (free-end) number (N.Tm), and nodes to termini ratio (Nd/Tm). A node (Nd) was defined as a point where three or more trabeculae were joined. A terminus (Tm) was defined as the free-end of a trabecula. Node-related values are indicative of interconnectedness, and terminus-related values indicated disconnectedness of trabecular network (32, 33, 34).

3.2 Observations in Clinical Specimens

Bone resorption and bone formation are opposite ends of the spectrum of bone metabolism. We usually observed both changes simultaneously in patients with metastatic skeletal lesions. Our morphometric data suggest that bone metastases due to breast cancer amplify remodeling in predominantly quiescent bone surfaces approximately 10 fold. The dramatic increase in bone resorption was uncoupled from the subsequent bone formation phase with a net bone deficit (Table 1). This confirms that the secondary osteoblast response seen in normal bone remodeling is almost always impaired in osteolytic bone lesions (6, 35) including breast carcinoma and myeloma (8).

Table 1. Bone histomorphometric results of 33 patients with bone metastasis due to breast carcinoma.

Morphometric parameters[a]		Controls (n=20)	Metastasis (n=33)
ES/BS	(%)	1.5±0.3	26.4±4.4[b]
OcS/BS	(%)	0.4±0.1	9.4±2.6[b]
N.Oc/BS	(/mm	0.1±0.1	1.7±0.5[b]
OV/BV	(%)	0.2±0.1	1.1±0.3
OS/BS	(%)	2.0±0.5	6.9±1.6
N.Ob/BS	(/mm)	1.5±0.3	2.7±0.5
Tb Th	(μm)	121.0±11.4	85.5±4.4[b]
BV/TV	(%)	20.3±1.1	13.2±1.0[b]
WoV/BV	(%)	0	11.9±4.3
TuV/TV	(%)	0	16.8±2.5

[a]Sections of the femoral bone biopsies from patients with histologically proven breast carcinoma with bone metastases were analyzed to determine the histomorphometric parameters (mean values ± SE are shown). [b]Significantly different ($p<0.05$) from corresponding controls (Mann-Whitney U-test). See text for abbreviations of bone morphometry parameters.

Patients with bone metastases are heterogeneous with respect to the amount of bone marrow replaced by tumor tissue, bone resorption rate and the secondary osteoblastic response. Our quantitative data suggest that bone resorption and bone formation are dependent on the stage of the metastatic lesions. Osteoclastic bone resorption was quantitatively increased at earlier stages and decreased when tumor volume was higher (Figure 5). We also noticed an increase in bone formation parameters at earlier stages followed by a reduction later on. Other morphometric studies on human tissue have also suggested a decrease in both osteoclast-mediated bone resorption and new bone formation at later stages of bone destruction (6, 35, 36). Similarly, morphometric data obtained from myeloma patients have shown tumor volume-related osteoclastic bone resorption and formation (37).

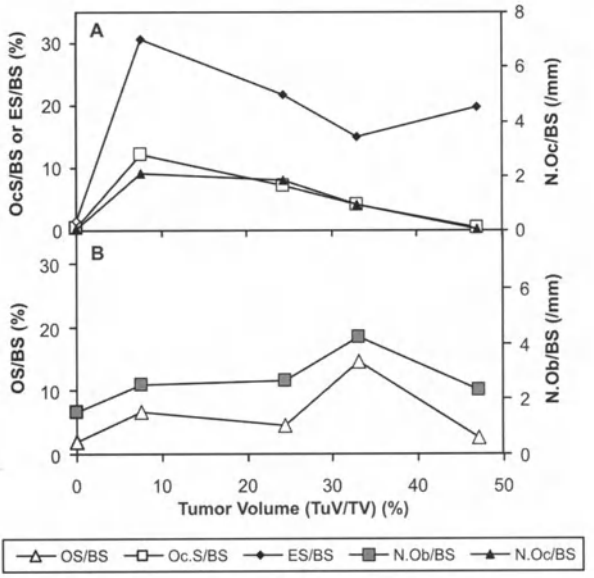

Figure 5. Biphasic osteoclastic bone resorption (A) and uncoupled bone formation (B). Tumor volume values were separated in 4 different groups with values 7.5%, 24.4%, 33%, and 47.1% respectively, to show trend of resorptive and formative parameters. Mean values and SE are shown in Table 1.

3.3 Observations in Experimental Models

Bone histomorphometry performed on metastases in animal models after intracardiac cell injection revealed significant increases in osteoclast-mediated bone resorption with a decrease in bone formation in femoral bone metastasis (Table 2). We also confirmed the morphologic observation that the MAT-LyLu spinal metastatic tumor induced a mixed, osteoblastic and osteolytic reaction, judged by the significantly increased parameters of both resorption and formation compared with controls (Table 3). Although we showed a net bone deficit, judged by the decrease in bone volume, bone histomorphometry confirmed that spinal metastasis in the MAT-LyLu model could be suitable for studying bone metastasis due to prostate carcinoma. Bone histomorphometry showed that MAT-LyLu femoral metastasis did not mimic the human situation in prostate cancer patients. However, our data obtained from spinal metastasis corresponded to previously reported morphometric evidence for bone resorption in prostate cancer patients with mixed, dominantly osteoblastic bone metastase (14).

Table 2. Bone histomorphometric results in the experimental femoral bone metastasis.

Morphometric parameters[a]	MDA-MB-231		B16/F1		Mat-LyLu	
	Controls (n=10)	Tumour (n=12)	Controls (n=10)	Tumour (n=12)	Controls (n=10)	Tumour (n=10)
ES/BS (%)	1.1±0.2	17.4±4.7[b]	0.8±0.1	6.0±1.5[b]	3.5±0.5	25.1±6.7[b]
OcS/BS (%)	0.2±0.1	6.5±1.8[b]	0.3±0.1	1.1±0.1[b]	1.6±0.26	9.7±2.1[b]
N.Oc/BS (/mm)	0.03±0.004	1.7±0.6[b]	0.1±0.01	0.7±0.1[b]	1.0±0.1	4.0±0.9[b]
OV/BV (%)	0.5±0.1	1.1±0.3	1.0±0.2	0.02±0.02[b]	0.6±0.03	0.6±0.3
OS/BS (%)	3.3±0.3	5.6±1.3	2.3±0.3	0.7±0.2[b]	1.8±0.2	1.5±0.4
N.Ob/BS (/mm)	1.9±0.2	1.8±0.5	2.0±0.4	0.7±0.2[b]	1.8±0.2	1.1±0.3
BV/TV (%)	29.5±0.3	21.2±1.1[b]	15.8±0.3	8.2±0.8[b]	21.4±0.7	18.2±2.2[b]
TbTh (μm)	104.6±7.8	78.8±10.6	62.2±7.5	32.5±2.6[b]	80.2±3.6	63.6±7.00[b]
TuV/TV (%)	0	30.3±4.1	0	77.7±18.1	0	43.8±7.5

[a]Longitudinal sections of the femora were analyzed to determine the histomorphometric parameters. [b]Significant difference ($p< 0.05$) between metastatic tumor group and corresponding control group (Mann-Whitney U test). Data represent mean ± SE of the combined values of all animals in each of the groups in three separate experiments using three different cell lines. See text for abbreviations of bone morphometry parameters.

Table 3. Bone histomorphometric results in the experimental spinal bone metastasis.

Morphometric parameters[a]	MDA-MB-231		Mat-LyLu	
	Controls (n=10)	Tumor (n=12)	Controls (n=5)	Tumor (n=5)
ES/BS (%)	0.9±0.2	14.9±2.2[b]	1.9±0.3	9.9±2.2[b]
OcS/BS (%)	0.2±0.1	6.5±1.6[b]	0.9±0.2	4.6±0.7[b]
N.Oc/BS (/mm)	0.04±0.01	2.0±0.7[b]	0.7±0.1	2.4±0.5[b]
OV/BV (%)	0.4±0.1	1.1±0.7	0.1±0.04	3.3±0.9[b]
OS/BS (%)	1.5±0.2	4.1±1.0[b]	1.0±0.3	3.3±1.0[b]
N.Ob/BS (/mm)	1.1±0.2	1.5±0.4	1.0±0.3	2.6±1.0[b]
BV/TV (%)	28.9±0.7	19.2±2.16[b]	24.0±0.8	17.5±0.9[b]
TbTh (μm)	108.9±20.0	77.1±8.0[b]	83.4±4.2	66.4±3.9
TuV/TV (%)	0	46.3±4.5	0	50.0±19.6

[a]Longitudinal sections of the spine were analyzed to determine the histomorphometric parameters. [b]Significant difference ($p<0.05$) between metastatic tumour group and corresponding control group (Mann-Whitney U test). Data represent mean ± SE of the combined values of all animals in each of the groups in three separate experiments using three different cell lines. See text for abbreviations of bone morphometry parameters.

Although MDA-MB-231 and B16/F1 tumors were both osteolytic, with significant increases in osteoclastic bone resorption parameters; there were some important differences between them. In the B16/F1 model, the tumor volume was 2.6 times greater compared to the MDA-MB-231 tumor while parameters of bone resorption were 3, 6 and 2.5 times lower for eroded surface, osteoclast bone surface and osteoclast number per bone surface, respectively. We suggested that the B16/F1 model was more suitable to

study the later stages of metastatic bone disease where more than 50% of total tissue was replaced by tumor cells. We also noticed a significant reduction in bone formation parameters in this model, corresponding to later stages of human bone metastasis. We have also found that the MDA-MB-231 model was more suitable for studying the earlier stages of bone metastasis where less than 50% of the total tissue volume was replaced by tumor cells (Figure 6).

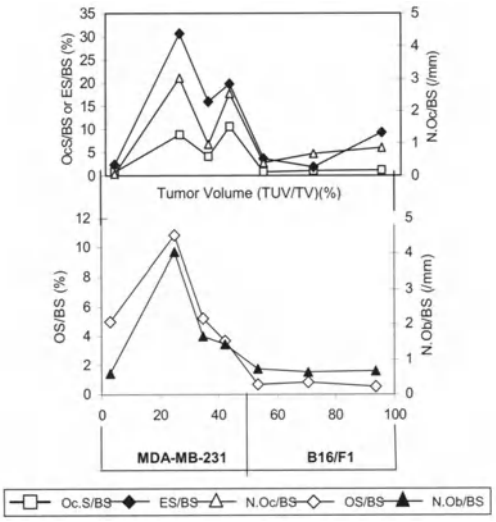

Figure 6. Comparative morphometric study on two in vivo models (MDA-MB-231 and B16/F1). Longitudinal sections of the femora were analyzed to determine histomorphometric parameters. Tumor volume values were separated in two groups (tumor volume <47% and tumor volume >47%). MDA-MB-231 tumor volume was ranging 0.4 to 42.5%. B16/F1 tumor volume was ranging from 46.3% to 95%. Mean values and SE are shown in Table 2.

Microstructural analysis based on node-strut analysis suggested different mechanisms of bone loss in the B16/F1 compared with MDA-MB-231 metastatic femoral tumor. The B16/F1 model was characterized by a significant increase in disconnectivity and irreversible bone loss with 50% reduction of bone volume. However, microstructural irreversible changes in the MDA-MB-231 bone model were manifested by a significant reduction in connectivity and 30% reduction of bone volume. Although the decrease in connectivity and increase in disconnectivity was found in the MAT-LyLu

model, these differences did not reach significant values (Figure 7). Data presented here suggest that there are morphometric peculiarities of the B16/F1 metastatic bone tumor with higher tumor volume and 2.9-fold lower eroded surface compared with the MDA-MB-231 model. Although bone resorption was significantly higher than in the corresponding controls, osteoclast surface was only 18% of the eroded surface compared to 40% in the MDA-MB-231 model. However, there was a significant reduction in bone volume in both models. We suggested that osteoclastic bone resorption was not a major factor and some other cells or mechanisms were responsible for bone resorption in the B16/F1 model.

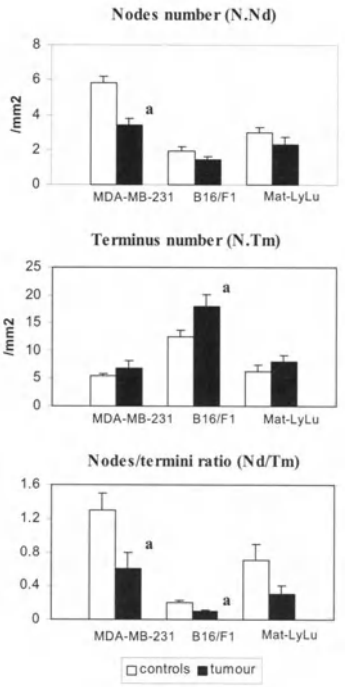

Figure 7. Results of node-strut analysis in experimental animal models. Longitudinal sections of the femora were analyzed to determine the microanatomical structural parameters described in Bone Histomorphometry and Node-Strut Analysis in the Assessment of Bone Metastasis. [a]Significant difference ($p<05$) between metastatic tumor and corresponding control group (Mann-Whitney U-test).

4. CONCLUSIONS

Bone metastases can be divided morphologically into three development stages: "phase of early appearance", "phase of interaction" and "phase of carcinomatosis" (8). Our quantitative data suggest that bone resorption and bone formation were dependent on the stage of metastatic bone disease in patients with metastatic breast carcinoma. Osteoclastic bone resorption was quantitatively increased at earlier stages and decreased when tumor volume was higher. We also noticed an increase in bone formation parameters at earlier stages followed by a reduction later on.

Osteoclastic bone resorption is widely accepted as quantitatively important in human metastatic breast carcinoma (7, 38, 39). Although some authors propose that tumor cells can be responsible for bone resorption, (7, 36) direct bone resorption by tumor cells is still controversial. There is in vitro evidence that breast carcinoma and prostate adenocarcinoma cells can directly degrade bone (40, 41). Tumor–mediated osteolysis by B16/F1 melanoma cells has been shown *in vivo* (27) and is described as a intertrabecular type of bone metastasis (2, 15, 16). We found that histomorphometric alterations in B16/F1 melanoma model corresponded to the later stage of metastatic bone disease where more than 50% of bone marrow was replaced by tumor cells. Data presented here indicated morphometric peculiarities of this model with higher tumor volume and lower eroded and osteoclast surface compared with the widely studied MDA-MB-231 osteolytic model. However, the B16/F1 melanoma tumor did not meet all of criteria for intertrabecular type of bone metastasis because of significant reduction in bone volume.

Bone histomorphometry techniques should serve to identify reproducible animal models of bone metastasis that mimic human bone metastasis. There is currently no animal model that fully reproduces the changes in human osteosclerotic bone metastasis. Our analysis showed site specific phenotypic differences in the murine Mat-LyLu prostate carcinoma model. Bone histomorphometry confirmed mixed type of bone metastasis in spine. However, femoral Mat-LyLu tumor was osteolytic. Data in the literature suggest that site specific differences exist showing phenotypic differences between subpopulation of osteclasts originated from intramembranous bone (calvaria) and enchondral bone (long bone) (42) leading to site specific differences between bone resorption and formation. In metastatic breast cancer patients, new bone formation is more commonly present in iliac bone compared to the femur (39).

Our morphometric data also suggested that MAT-LyLu spinal bone metastasis may be a suitable model to study metastatic prostate cancer. Although prostate metastases are bone-forming, at some point of time,

osteoblastic bone formation is preceded by osteoclastic activation (25). This concept may be very important because of its potential implications for treatment with osteotropic antiresorptive agents. Similarly, comparison between fracture and non-fracture groups of metastatic patients by histomorphometry and node-strut analysis of biopsy material might also be useful for direct assessment of the effect of osteotropic drugs for prevention of bone fracture and improvement of quality of life in cancer patients.

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Chapter 4

CLINICAL MODALITIES FOR THE DIAGNOSIS, CHARACTERIZATION AND DETECTION OF BONE METASTASES

Michael H. Weber[1,4], Jonathan C. Sharp[4], Thomas H. Hassard[2], Martin Reed[5], James A. Thliveris[3], F. William Orr[1]

[1]The Department of Pathology, University of Manitoba, Winnipeg, Manitoba, Canada; [2]Department of Community Health Science, University of Manitoba, Winnipeg, Manitoba, Canada; [3]Department of Human Anatomy and Cell Science; [4]Institute for Biodiagnostics, National Research Council of Canada, Winnipeg, Manitoba, Canada; [5]The Department of Radiology, Health Sciences Centre, Winnipeg, Manitoba, Canada

1. INTRODUCTION

1.1 Clinical Significance

Breast cancer is a leading cause of death in women, occurring in one of eight during their lifetime. A comparable cancer in men, prostate cancer, claims the lives of just under 30,000 men in the United States each year with a prevalence of one in six (1). These tumors, along with carcinomas of the lung, thyroid, and kidney have a high propensity to metastasize to bone, which constitutes one of their most serious complications, creates a great challenge for treatment, and often carries a poor prognosis. Metastases are the most frequent bone tumors and cause significant morbidity due to pain, osteolysis, pathological fractures, hypercalcemia, and anemia (2). Established bone metastases are resistant to treatment and current therapeutic approaches such as endocrine therapy, radiation therapy, and chemotherapy are often ineffective (3). This chapter has been written on the premise that a

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better understanding of the pathophysiology of bone metastasis may provide insight into the design of diagnostic techniques that would help to identify bone metastasis formation at its earliest stages. This may provide an opportunity for early treatment and alleviate the necessity to treat them at a late stage.

1.2 Pathophysiology

The majority of bone metastases arise from a hematogenous route. The venous system, as opposed to the arterial system is especially important in Batson's paravertebral plexus. The axial skeleton and proximal long bones are two sites with high propensity for metastatic involvement because of Batson's plexus communications and the greater vascularity of red marrow as opposed to yellow marrow found in these anatomic locations (4, 5).

There is strong evidence that the formation of bone metastases is the result of a synergistic relationship between cancer cells and the unique environment within the osseous microcompartment (Figure 1). Osteolysis is a dominant feature of most established metastases and is widely recognised to be the consequence of osteoclastic bone resorption. Many cancer cells secrete parathyroid hormone-related protein which will promote the osteoblasts to initiate osteoclastogenesis utilizing the receptor activator of NF-κB (RANK) and its ligand (RANKL) (6, 7). The multinucleated osteoclasts, derivatives of their monocytic precursors, resorb bone, releasing growth factors and cytokines, including transforming growth factor-β (TGF-β), which in turn can stimulate proliferation of metastatic cells via Smad and MAP kinase pathways, reinforcing this pathological cycle (8). There is evidence that some metastatic cells can induce bone resorption directly by secreting matrix metalloproteinases (9).

Cells from prostate cancer can induce osteosclerosis. Mediators of osteoblastic reactions include endothelin-1, TGF-β, fibroblast growth factor and endothelial growth factor (EGF) which can stimulate osteoblasts to initiate bone formation [reviewed by Guise et al., 2003 (10)]. A second pathway involves secretion of proteinases, prostate specific antigen and urokinase plasminogen activator, which can activate latent TGF-β and inactivate parathyroid hormone-related protein (11, 12).

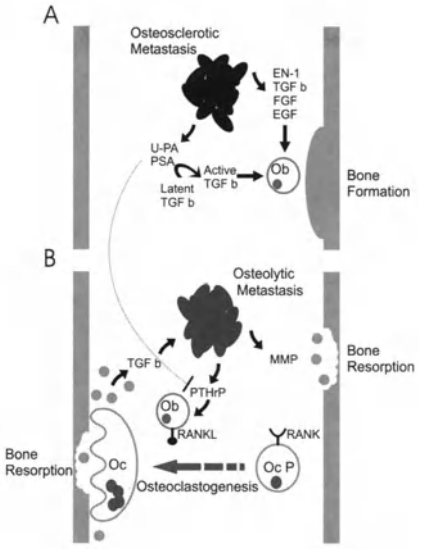

Figure 1. Current concepts relating to the pathophysiology of bone metastases. A diagrammatic representation of bone metastasis induced (A) osteosclerosis and (B) osteolysis. Parathyroid hormone-related protein (PTHrP), Receptor activator of NF-κB (RANK), Receptor activator of NF-κB ligand (RANKL), Matrix metalloproteinases (MMP), Endothelin-1 (EN-1), Transforming growth factor-β (TGFβ), Fibroblast growth factor (FGF), Endothelial growth factor (EGF), Prostate specific antigen (PSA), Urokinase plasminogen activator (U-PA), Osteoblasts (Ob), Osteoclasts (Oc), and Osteoclast precursor (OcP).

1.3 Detection

Insightful interpretation of results from various detection techniques is dependent on understanding the pathophysiology of bone metastasis development. Metastatic bone disease ranges from colonization of the marrow by micrometastatic cells to clinically significant bone metastases and resulting changes to bone microanatomy. Single micrometastatic epithelial cells in a mesenchymal bone marrow cavity are distinguishable using immunocytochemistry, flow cytometry and molecular techniques. Biochemical markers may be used to directly detect and monitor changes in normal bone metabolism due to the effects of metastases on the normal balance between bone formation and resorption. Diagnostic imaging techniques such as plain radiography, bone scintigraphy and magnetic resonance imaging directly identify late stage clinically significant metastases and the resulting change in the bone architecture.

The detection of bone metastases is intimately dependent on the pathophysiology of the primary tumor. The three modalities investigated in this chapter for the detection, quantification, and further understanding of the development of bone metastases and response to therapy include the use of tumor specific molecular markers, identification of serum levels of biochemical markers, and radiological identification.

2. MOLECULAR MARKERS

Bone marrow and blood are both relatively accessible and have been frequently surveyed for the presence of disseminated tumor cells in patients with solid tumors. Blood represents the vehicle for the hematogenous spread of disseminated tumor cells to bone (13). Bone marrow acts as a filter and is a preferential environment for the eventual growth of disseminated tumor cells (14). Small aggregates of tumor cells have been observed using standard cytologic and histologic methods (15) and some workers have identified single disseminated tumor cells (16). The cellular aggregates may have greater malignant potential than single disseminated tumor cells (17-19, 20). Despite the cross reactivity of membrane antigens with hematopoietic cells, immunocytochemical tests using monoclonal antibodies to epithelial-specific antigens have been created to identify disseminated tumor cells in the bone and peripheral blood (21-23). The non-epithelial nature of blood and bone marrow make it ideal for the differentiation of epithelial derived tumor cells using epithelial-specific proteins (23, 24), RNA expression (25-27), and tumor-specific DNA abnormalities (28, 29). For a review, see Zippelius and Pantel (30).

The goal of most detection techniques is good sensitivity and specificity. To improve specificity the marker must not be present in the background hematopoietic cells. However using both tumor specific antibodies (31) and RT-PCR (32, 33) false positives have been reported. Quality control is key to decreasing crossover contamination and amplification of non-specific and pseudogenes. To improve sensitivity, a number of markers may need to be screened to avoid false negatives (34-36).

The current convention is to use molecular markers to identify disseminated tumor cells (37, 24) as well as to characterize these cells for an improved knowledge of the pathophysiology of bone metastases (38). These approaches have led to the identification of critical proteins and/or genes involved in bone metastasis development. Disseminated tumor cells, collected from cancer patients, have a metastatic phenotype (39), elevated proliferation potential (40) and can generate metastases in mice (41). The ability of disseminated tumor cells to act as seeds for clinically significant

metastases is substantiated by both the long dormancy of residual cancer cells and by the transmission of cancer after organ transplant (42, 43).

However, the presence of disseminated tumor cells in blood or marrow does not always predict the eventual formation of clinically significant bone metastases (44). It has been demonstrated with follow-up sampling that there is a clearance of disseminated tumor cells after surgery (45). Moreover, it is likely that the majority of disseminated single metastatic tumor cells in bone do not proliferate (46). Only a few large long-term follow-up studies have been carried out (44, 47). As a prognostic indicator, the presence of disseminated tumor cells is at best comparable to established staging techniques (48). In addition, the number of patients with disseminated tumor cells varies from study to study (49, 50). A standard detection technique is critical for the accurate comparison of studies (51). For example the prognostic significance of disseminated tumor cells in blood may (52) or may not (24) be comparable to that of bone marrow. This may in part be due to the difference in definition of prognosis from the number of patients with disseminated tumor cells who will develop clinically significant metastases to the five-year survival rates. Sampling time with reference to when the surgical procedure is conducted is crucial, affecting both the number and characteristics of the disseminated tumor cells (45, 53). To reduce sampling error more than one sample is required to accurately test for the presence and characteristics of scarce and inhomogeneously localized disseminated tumor cells (54).

3. BIOCHEMICAL MARKERS

Biochemical markers expressed by neoplastic cells, such as carcinoembryonic antigen (CEA), tissue polypeptide antigen (TPA), and the carbohydrate antigen (CA 125), have been used to identify patients with a number of primary tumors that have a propensity to metastasize to bone including breast, ovary, lung, and prostate cancer. Some of these markers have been used to monitor the response of bone metastases to therapy (55-57). Regretfully, the ability of CEA, TPA and CA 125 to diagnoses bone metastases has been inconsistent when compared with markers for bone turnover (58, 59). Prostate specific antigen (PSA) is a widely used biochemical marker, not only for diagnosis and follow-up of prostate cancer, but also for evidence of prostatic bone metastases (60, 61). A level of PSA more than 100 µg/l indicates a high probability of bone metastases, however the sensitivity of this cutoff is inconsistent, from 42.9% to 83.3% (62, 63, 64).

Biochemical markers can be used to detect and monitor changes in bone metabolism due to the effects of metastases in the bone environment. Some markers appear early in the development of the bone metastasis and may be effective adjuvant monitoring techniques for either osteolytic (Table 1) and or osteoblastic (Table 2) bone metastases. Biochemical markers for bone metabolism such as alkaline phosphatase (ALP), and more recently, specific markers for either bone formation or resorption, have been instrumental in deciphering the pathophysiology of collagen and bone metabolism and therefore the evaluation of bone metastases [reviewed in (65-67)].

Table 1. Bone Resorption Markers

Source	Markers	Ref
Type I collagen degradates	Pyridinium cross-links (urine pyridinoline (PYP), deoxy-pyridinoline (DPD))	(124, 125)
	Pyridinium cross-linked collagen peptide fragments (C-terminal telopeptide (CTx), N-terminal telopeptide (NTx)	(126, 76)
	Hydroxyproline	(127)
Non-collagenous protein	Bone sialoprotein (BSP)	(128)
Osteoclast enzyme	Tartrate-resistant acid phosphatase (TRAP)	(129)

Table 2. Bone Formation Markers

Source	Markers	Ref
Type 1 procollagen propeptides	C-Terminal propeptide fragment (PICP)	(130)
	N-terminal propeptide fragment (PINP)	(130)
Alkaline phosphatase	Serum alkaline phosphatase (ALP)	(131, 132)
	Bone alkaline phosphatase (BLP)	(130)

The seeding of bone by metastatic cells disrupts the normal homeostatic balance between bone resorption and formation (9, 68). In late stage breast cancer metastases to bone, osteolytic markers are increased, compared to patients responding to treatment and patients with indolent bone metastases which have low osteolytic markers (69) and high osteoblastic markers (70, 71). Although, prostatic bone metastases are mainly osteoblastic (osteosclerotic), both osteoblastic and osteoclastic activities can be present (72). Newer non-collagenous serum proteins include periostin (73), and

interleukin-6 (74), and –18 (75). The sensitivity of biochemical markers still limits their popular use clinically for the early diagnosis of bone metastases (76). Bone metabolic markers are affected by menopause (77) and therefore biochemical levels can be influenced by age, chemotherapy and hormone therapy. Markers of bone resorption (Table 1) and formation (Table 2) have been used to monitor the effects of bisphosphonate treatment and can be applied to individualizing the therapy regimen (78). A reduction in bone pain with bisphosphonate treatment was found to be directly proportional to a decrease in bone resorption markers (78). Lastly, studies demonstrating biochemical markers as independent prognostic indicators of survival and longitudinal studies on their application to monitoring therapy have yet to be conducted.

4. RADIOLOGICAL IMAGING

Prior to the 1970's, the diagnosis of bone metastases depended upon nonspecific findings such as pain, increased serum alkaline phosphatase, and hypercalcemia, in combination with radiography. Plain radiography is relatively insensitive, since a minimum change in bone of approximately 30% is required for the detection of a lesion (79). In addition, degenerative bone changes, osteopenia and inaccurate examinations have lead to missed diagnosis of metastatic bone lesions.

The development of the gamma camera and $^{99}Tc^{m}$-phosphorus compounds in the 1970s revolutionized bone scintigraphy and the detection of bone metastases (80, 81). Since then, computerized tomography (CT) and more recently magnetic resonance imaging (MRI) have come forward to rival bone scintigraphy for the detection of bone metastases.

4.1 X-ray

Despite its limitations, plain radiography is still used in clinical practice to confirm other imaging studies and to investigate symptomatic sites. The most significant limitation is that not all patients with bone metastases present with lesions on plain radiographs. Commonly accepted reasons for these limitations are the variations in film exposure between follow-up studies, overlying gas, the size of the patient, location of the lesion (sacrum, pelvis, spine) and discrepancies in lesional size.

It is commonly accepted that a period of six to eight months is required between examinations before changes in the appearance of bone metastases become evident with plain radiography. Because of poor sensitivity, radiography is typically not used as a screening method. Plain radiography is

adequate for true osteolytic lesions and therefore useful to assess fracture risk, however the method is less useful to identify mixed lytic-blastic metastases (82).

4.2 Computed Tomography (CT)

Unlike radiography, computed tomography (CT) is not limited by low contrast resolution and by the obscuring effect of superimposed bone and soft tissue. CT offers a higher sensitivity (83) and earlier detection of lytic changes in trabecular bone than radiography (84). On CT, bone metastases have an increased attenuation value compared to the fat containing marrow, and because of this, CT is an effective modality for the differentiation between benign and malignant lesions observed on bone scintigraphy (85). Although CT has been reported to be an effective means of evaluating bone metastases in the skeleton because of its sensitivity compared to plain film x-ray (86, 87), CT is a very awkward method for screening of the entire skeleton.

4.3 Bone Scintigraphy

Bone scintigraphy is twice as sensitive as radiography (88) and enables the scanning of the entire skeleton in a short period of time. Bone scintigraphy is used as a screening technique for the detection of bone metastases primarily due to its high sensitivity and acceptable specificity (59). However, $^{99}Tc^{m}$-phosphorous compounds do not specifically associate with tumor cells or offer information on the osteolytic process. Instead these complexes are incorporated into hydroxyapatite crystals as a result of new bone formation, a nonspecific response of normal bone to a pathological process. A metastatic lesion, a fracture, infection, inflammation, or degenerative change, typically appears as areas of increased uptake compared to the normal surrounding bone.

Rigorous evaluation of the disease process is limited and only progression can be identified reliably with bone scintigraphy. However, as with plain radiography, a lag time of six to eight months is also required before sufficiently gross changes will become evident with scintigraphic findings (89, 90). The use of bone scans for assessment of the response to therapy is unreliable, primarily due to the flare phenomenon, which is a consequence of an initial increase in bone formation in patients responding to systemic therapy (91). Bone scintigraphy cannot differentiate between tumor progression and the flare phenomenon (92) or provide information on the extent of individual lesions.

4.4 Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging (MRI) provides a digitized image with contrast based upon the hydrogen nuclei concentration (proton density) and proton interaction with neighboring molecules (T1 and T2 relaxation)[1]. Hydrogen protons are most plentiful in water and fat. Water and fat have opposing signal intensities and as such on T1-weighted images, fat gives a high signal and water a low signal, while on T2-weighted images, water offers a high signal and fat a low signal. Pathology, such as bone metastases, changes the relative water and fat concentration, thereby producing contrast (93, 94).

MRI is the most sensitive and only imaging modality to allow direct visualization of the marrow (95). MRI can discriminate between bone metastases and non-neoplastic lesions, such as osteoporotic compression fractures, degenerative lesions (96-98), and inflammatory responses. MRI can detect bone metastases that are not apparent on radioisotope bone scans because it can detect an intramedullary lesion before destruction of the cortex, and before an osteoblastic reaction is detected on bone scan (99, 100). In particular, MRI is superior to planar scintigraphy in its ability to detect spinal metastases due to the difficulty of planar scintigraphy to recognize subtle radionuclide abnormalities (101, 102). More recently, single photon emission computed tomography (SPECT) has been shown to be more beneficial in the detection of single vertebral metastases than planar bone scans (103), and argued by some authors to be comparable to MRI (104).

The sensitivity of MRI is high, so to improve specificity, an appropriate choice of an acquisition pulse sequence is required. Spin-echo offers good signal-to-noise ratio and contrast of bone and metastatic lesions. The need for rapid and effective sequences to survey the entire skeleton for signs of metastases has resulted in fast spin-echo and inversion recovery image sequences development (105). Our published studies have demonstrated that high resolution MRI is an effective means for the quantitative evaluation of the normal variation in bone microanatomy as compared to a histological "gold standard", and a superior means of quantification in comparison to micro x-ray (Figure 2) (106).

[1] T1 and T2 relaxation are defined as the time required for the excited protons to return to equilibrium and diphase, respectively (133).

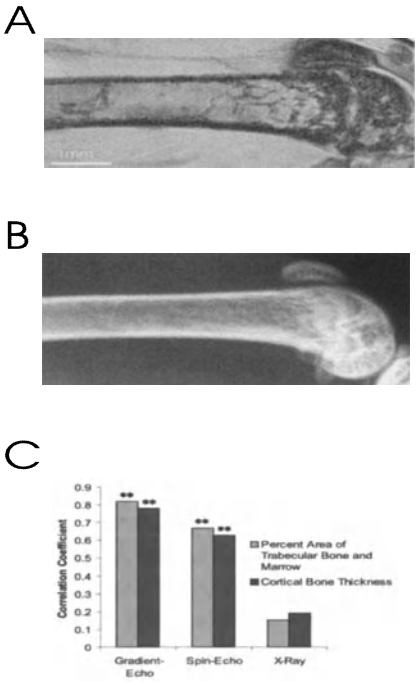

Figure 2. Representative images of a distal femur from a C57bl/6 mouse. (A) Longitudinal gradient-echo image from an 11.7T MR microscopy scanner with in-plane resolution of 35µm. Note the detail MRI provides of the marrow micro-architecture. (B) High-resolution x-ray of a distal femur from a C57bl/6 mouse. (C) Details of a comparison between cortical bone thickness, percent marrow and trabecular bone data from the histological gold standard and data from an 11.7T MR microscopy scanner and x-ray. * and **indicate significance between quantified MRI and histological data at $p \leq 0.01$ and $p \leq 0.001$, respectively, by a Multiple Regression test.

In the human adult, the majority of the marrow is yellow or fat-containing. On T1-weighted spin-echo MR images, fat has high signal intensity, and water and bone metastases low signal intensity (except for some rare lipoblastic metastases). Bone metastases can often be differentiated from low intensity focal areas of red hematopoeitic marrow on T1-weighted MR images since red marrow has a lower signal intensity than fat but a higher signal intensity than normal muscles, is normally uniformly demarcated, and can have centrally located fat (107).

The difference in resonance frequency between water and fat protons can be useful for the detection of metastases. Bone metastases appear using a fat-suppressed T1-weighted image as mixed to high intensity (108). Short tau

inversion recovery (STIR) and out-of-phase acquisitions improve the contrast between metastases and normal bone marrow (109).

Bone metastases are significantly brighter than background marrow with T2-weighted spin-echo images due to the higher water content in these lesions. In addition, bone metastases can have a diagnostic rim of bright T2 signal, forming the circumference of the metastases (107). Diffusion weighted spin-echo imaging is reported to be no better than non-contrast enhanced T1 weighted imaging, but is advantageous over T2 weighted imaging (110).

Gradient-echo sequences show metastases as more homogeneous and higher in signal intensity than normal marrow, and also enable direct visualization of the resultant changes in bone. Because of a lack of mobile protons, trabecular bone offers no detectable signal and generates local field heterogeneity. The heterogeneous magnetic field has little effect on spin-echo sequences because of the correcting 180° pulse. The gradient-echo sequence cannot compensate for field heterogeneity thus leading to signal cancellation and loss (111-114). As the echo time (TE) is lengthened, signal can decrease markedly in regions of bone with increased trabecular bone. This is of clinical relevance when trabecular lysis occurs as a result of metastatic disease since the signal will become higher than the rest of the bone.

In an attempt to improve on this, a number of contrast agents have been used. There is no observable (<10%) change in normal marrow with the injection of gadolinium chelates on T1-weighted images (115, 116). Bone metastases typically have a strong signal increase with the injection of gadolinium chelates, however this is not pathognomonic because a similar change is seen in infectious or inflammatory reactions. Supermagnetic iron oxide particles are taken up by macrophages and markedly decrease the T2 in normal marrow (117). However, in neoplastic bone marrow there is only a minor or no signal decline (118).

5. CONCLUSION

Metastatic bone tumors are the most common type of malignant bone lesion, and bone is the third most frequent metastatic site after the lung and liver (119). The molecular mechanisms responsible for the spread and survival of metastatic tumor cells are unclear. Ideally, these single micrometastatic cells would represent preferable targets for treatment, compared to established late stage bone metastases where there is a much larger tumor cell population and extensive alteration in the bone architecture. Hypothetically, therapeutic intervention at this early stage would benefit

from targeting dormant single cells as well as organized proliferating aggregates of tumor cells. Nevertheless, the mechanisms responsible for the dormancy or growth of disseminated tumor cells in the bone microenvironment are not fully understood. Arguably, there is a dormant period between the seeding of tumor cells to bone marrow and the later development of clinically significant bone metastases. However, it is not known what proportion of micrometastatic tumor cells in the bone marrow are precursors of clinically significant metastases and, because of their dormant states, these cells are resistant to many chemotherapeutic modalities. A comparison of the different modalities used for the detection of bone metastases has been discussed here to better understand the pathophysiology and to investigate the strengths and weaknesses of the respective techniques.

Research towards the molecular basis of bone metastases has identified a number of proteins involved in the process. Molecular markers can be used to both directly and indirectly identify the presence of minimal residual disease; however the standardization of protocols is still required for reproducibility and reliability before routine clinical use. Bone biochemical markers are also not recommended for routine clinical use and further research is required to define their potential role in the early diagnosis of bone metastases, assessment of disease progression and response to therapy, and finally, rate of bone loss and fracture risk.

Current radiological techniques are non-invasive and can directly identify late metastatic disease and the effects on bone beyond simple bone densitometry. Standard plan radiography is used for painful areas or to check abnormalities with bone scintigraphy. Bone scintigraphy is an effective radiological technique for studies of the entire skeleton with a reduced false-negative rate. Quantification of metastatic involvement identified using bone scintigraphy is difficult, first because of the multiple disease sites involved, and second, the variation in which data on the response to therapy and progression is collected (120). MRI is sensitive with a high degree of accuracy and precision with regards to the intricacies of both the bone integrity and metastasis itself. The use of high resolution MRI, target specific contrast agents and large-scaled trials with more statistical power will better the future diagnosis, characterization and detection of bone metastases.

Unlike MRI, plain radiography and markers of bone metabolism do not contribute to the early diagnosis of bone metastases (121). However, when used together, the biochemical marker PSA and radiological techniques like MRI have have potentially better sensitivity for early diagnosis of bone metastases from prostate cancer than bone scintigraphy (122). Molecular markers of osteoclastogenesis, osteoprotegerin (OPG) and the soluble form

of the receptor activator for nuclear factor (NF)-kappaB (RANK-L), have been suggested as surrogate biochemical markers of tumor-induced osteoclastogenesis (123). Integration and standardization of these methods into a prognostic/predictive clinical algorithm is critical for significant improvement in the results of bone metastasis therapy.

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Chapter 5

METASTASIS IN THE BONE MARROW MICROENVIRONMENT

Paolo Bianco[1,2,4], Mara Riminucci[2,3] and Pamela Gehron Robey[4]
[1]Dipartimento di Medicina Sperimentale e Patologia, Universita "La Sapienza," Rome, Italy, [2]Parco Scientifico Biomedico San Raffaele, Rome, Italy, [3]Dipartimento di Medicina Sperimentale, Universita dell'Aquila, L'Aquila, Italy, [4]Craniofacial and Skeletal Diseases Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Department of Health and Human Services, Bethesda, MD

1. INTRODUCTION - BONE MARROW METASTASIS

Although metastatic growth of non-skeletal cancer in bone as an organ is commonly referred to as *bone* metastasis, any metastasis to bone is in fact a metastasis to the bone marrow. The extensive changes induced by cancer metastasis in bone (either lytic, or more rarely, sclerotic) represent secondary changes induced to fulfill the need of cancer cells for their anti-homeostatic growth. A common biological paradigm – "the seed and the soil" - is used to account both for the mechanism of bone metastasis (1), and for the mechanism of seeding of the bone marrow cavity by normal hematopoietic progenitor cells. In both instances, blood-borne cells must colonize the stromal framework of the bone marrow, that is, they must recognize and exploit a microenvironment suited for their proliferation. Circulating cells capable of growing in the bone marrow environment represent the seed. Characteristics of the bone marrow stroma that are conducive for the establishment of a bone metastasis provide the soil.

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2. THE NORMAL BONE MARROW STROMA IN VIVO

The bone marrow stroma is a complex three-dimensional network of cells defining a mesh of extracellular spaces where hematopoietic cells normally lodge, proliferate and differentiate (2). Several unique characteristics distinguish the bone marrow stroma from the stroma of most other organs. First and foremost, the stromal framework itself is mostly made of cells, rather than of extracellular matrix. Second, the extracellular matrix is remarkably poor of collagen, and enriched in hyaluronic acid and proteoglycans. Third, the local circulation includes an extensive sinusoidal section, in which blood flow slows down remarkably, allowing for margination of blood-borne cells (3). The inherent permeability of endothelial cells is conducive for transcellular (not intercellular) migration of circulating cells that have come to adhere to the endothelial surface. Marrow sinusoids do not feature a physically continuous basement membrane. An additional aspect makes the local circulation in the bone marrow a critical factor in defining a "soil" for cancer cells. That is, the bone and marrow circulation are connected through the sinusoidal network, and temporary changes in the direction of flow may occur between bone and marrow. The existence of a true portal-type circulation has long been postulated in bone, although its precise definition has remained elusive (3). It is important to note however, that local remodeling events may significantly affect the local concentration of extracellular calcium, and the circulatory link between bone and marrow may mediate the exposure of cancer to an extracellular milieu which may contain a variable tone not only of bone derived factors, but of calcium itself.

The extravascular compartment of the bone marrow includes multiple "cell phenotypes", which largely represent modulations of a single cell type (2, 4). Reticular cells [adventitial reticular cell, Westen-Bainton cells (5, 6)] are the dominant and most unique cell type in the bone marrow stroma. They extend over the abluminal side of sinusoids, and project from there into the hematopoietic cords. Here, they establish close physical contacts with individual hematopoietic cells. Upon maturation in the extravascular space of the bone marrow, hematopoietic cells leave the abluminal coating of reticular cells, and traverse endothelial cells to enter the circulation. Reticular cells are noted for membrane-bound alkaline phosphatase activity (7). Adipocytes are variably represented in the post-natal bone marrow, depending on age, site, and extent of the local hematopoietic activity. They do not represent a separate cell type, but are the product of a phenotypic conversion of individual reticular cells themselves (8). Osteoblasts (the mature, active bone-forming cells) and bone lining cells (the inactive,

quiescent ex-osteoblasts covering inert bone surfaces) are part of the bone marrow stroma and cooperate in the maintenance of the hematopoietic microenvironment. Macrophages are functionally a part of the physical substrate of the hematopoietic microenvironment, hence in a way they can be seen as part of the stroma, if the stroma is the substrate of hematopoiesis. However, they are excluded from the stromal system by their hematopoietic rather than mesenchymal origin. Smooth muscle cells are confined to the subendothelial layer of arterial capillaries, and to the tunica media of the marrow arteries (9).

Understanding the mechanism leading to the establishment of bone metastasis requires a definition of the sequence of physical events that must occur. These include: a) the presence of a sufficient number of cells in the blood perfusing the marrow that possess an inherent ability to seed the marrow; b) their margination in sinusoids; c) their adherence to the lumenal membrane of endothelial cell by specific arrays of adhesion molecules; d) their migration across the endothelial barrier; d) their adherence to the surface of stromal cells in the extravascular space of the bone marrow; e) their growth thereupon.

3. TROPISM FOR BONE AND OSTEOMIMESIS

Inherent characteristics of cancer cells that confer tropism for the bone marrow microenvironment have not been elucidated. Over the past few years, however, the expression of certain proteins characteristically enriched in the bone matrix, and normally expressed in a restricted fashion in osteoblasts, has been observed in cells from a variety of human epithelial cancers (10, 11). Expression of bone sialoprotein (BSP) (12) has been linked to the likelihood that epithelial cancers will metastasize to bone. The mechanism underlying this association has not been elucidated. Since BSP binds to bone mineral, it was proposed that it would mediate the attachment of cancer cells to the mineralized bone matrix. However, a continuous cellular investment separates the bone matrix from the marrow space where metastatic cancer cells lodge. Additional osteoblast-like features in epithelial cancer cells notably include the expression of the pivotal regulator of osteogenic differentiation, Cbfa1/Runx2, which is also involved in the regulation of matrix metalloproteinases used by cancer cells to degrade and invade the extracellular matrix. The expression of BSP and other proteins characteristic of bone may thus be seen as part of an osteomimetic phenotype, which includes the expression of matrix degradative properties in cancer cells, and may per se confer tropism for bone as part of the general invasive potential of cancer cells (13).

4. STROMAL CHANGES IN BONE METASTASIS

Few studies address in detail the early stages of the metastatic process in the human bone marrow. Analysis of human bioptic or surgical material taken from clinically overt metastasis do not help in this regard, since only the outcome of the process is apparent under these circumstances. Conversely, studies on iliac crest biopsies taken from cancer patients being investigated for hematological derangements or allied systemic disturbances may depict a sub-clinical stage of marrow colonization by blood-borne cancer cells.

Cancer cell emboli are easily detected in specimens of sub-clinical bone metastasis (Figure 1). In the extravascular space, cancer cells establish a physical relationship with stromal cells (Figure 2) which is distinct from the one observed with hematopoietic cancer (leukemia, lymphoma, myeloma). Hematopoietic cancer cells retain the same type of physical interaction with the stroma as normal hematopoietic cells. Stromal cells' processes surround individual cancer cells like they surround individual hematopoietic cells.

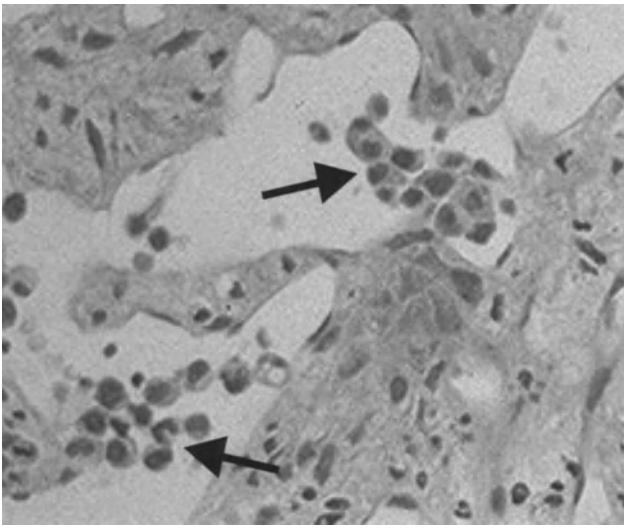

Figure 1. Cancer cell emboli in the bone marrow sinusoids.

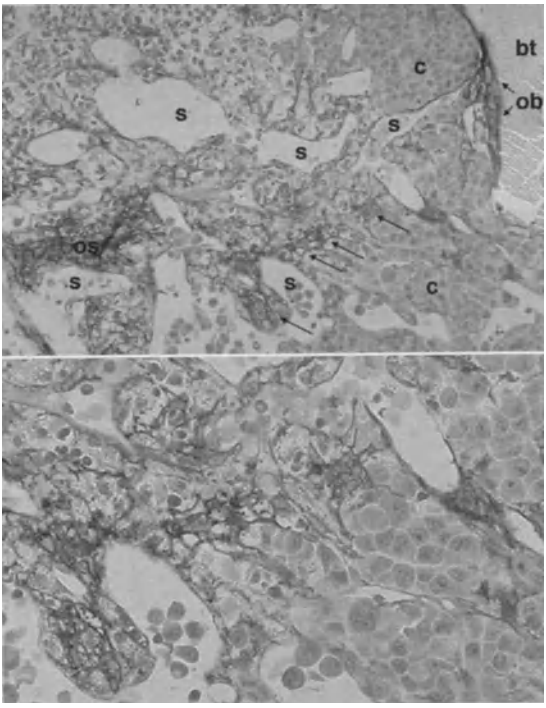

Figure 2. Stromal changes in the bone marrow following establishing of a subclinical bone metastasis from breast cancer. Iliac crest biopsy. Low temperature processed glycol methacrylate section reacted for ALP. ALP reactivity (shown in black) is found in osteoblasts on the surface of a bone trabecula (bt), in reticular cells in the bone marrow, and in a nascent osteogenic site within the bone marrow space. Reticular cells in the bone marrow stroma merge with the osteoblastic layer at the bone surface, but form a distinct adventitial layer at the ablumenal side of the marrow sinusoids. At multiple sites, a distinct crowding of reticular cells is observed over the outer aspect of the sinusoids (arrows). The nascent osteogenic site (os) within the marrow is also located at the ablumenal side of a sinusoid. s, sinusoid; ob, osteoblasts; bt, bone trabecula; os, nascent osteogenic site within the bone marrow.

With epithelial cancers, in contrast, solid masses of cancer cells are observed, from which stromal cell processes are excluded (Figure 3). As a result, the haphazard distribution of reticular cell processes among hematopoietic cells seen in the normal (or leukemic) marrow (6) is not observed in the presence of cancer cells, and the native location of stromal cells over the outer aspect of marrow sinusoids becomes as apparent as it otherwise never is under normal circumstances. The alkaline phosphatase reaction (which provides a marker of reticular cells) decorates the outline of the marrow sinusoids running among cancer cell masses in the marrow space. In addition, adventitial reticular cells undergo focal proliferative

events in the presence of cancer cells, generating local nests of stromal cells distributed along the course of the marrow sinusoids (Figure 2). Importantly, it is within these foci of stromal cell proliferation that the earliest osteogenic response of the marrow stroma to cancer becomes apparent. Fully mature osteoblasts differentiate within the bone marrow from the proliferating stromal cells crowded at the outer aspect of the sinusoids, and deposit recognizable osteoid (Figure 2 and 4). Interestingly, in addition to the stromal proliferation, a constellation of TRAP-positive macrophages can be observed along the outer aspect of the sinusoids (14). Since bone marrow macrophages normally do not express TRAP activity, TRAP activity may either be induced in resident macrophages by cancer cells, or the TRAP-positive 'macrophages' may represent immediate precursors of osteoclasts that are ectopically located within the marrow space proper, and away from bone surfaces. Perhaps surprisingly, an osteogenic response within the bone marrow stroma is an almost universal feature of early bone metastases, and is associated with the induction of bone resorption, regardless of the propensity of the specific cancer to generate a lytic or sclerotic type of bone metastasis. This illustrates how the general subdivision of bone metastasis in lytic and sclerotic only applies to late stages of the metastatic process, and only describes the ultimate prevalence of either bone resorption or bone formation. Both formation and resorption are tightly coupled in the early stages of development of bone metastases.

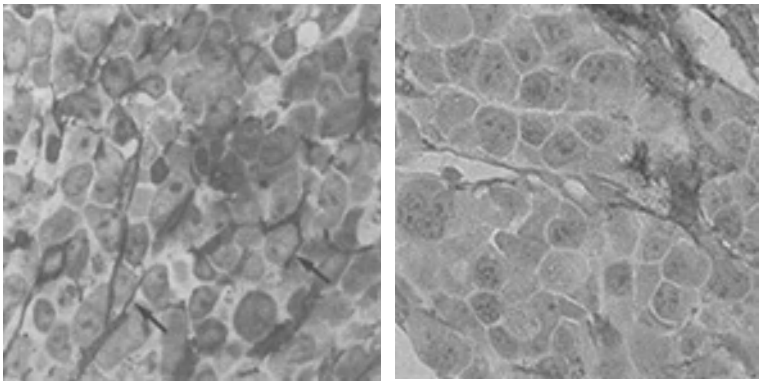

Figure 3. Low temperature processed, glycol methacrylate sections of human bone marrow, reacted for alkaline phosphatase. a) Acute non-lymphoblastic leukemia, b) breast cancer metastasis to the bone marrow. Reticular cell processes surround individual leukemic cells (a, arrows). In contrast, epithelial cancer cells form solid sheets which exclude reticular cell processes (b).

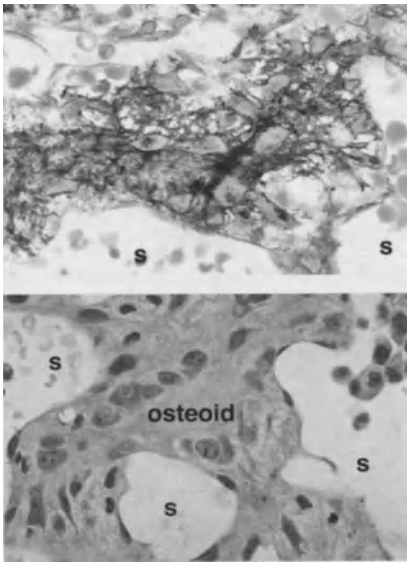

Figure 4. Differentiation of osteoblasts and deposition of osteoid within the bone marrow, in the presence of breast cancer metastasis. Detail of the nascent osteogenic site depicted in Figure 2. a), ALP reaction, b) H&E staining of a consecutive section, showing morphologically typical osteoblasts and appearance of osteoid between them

5. MARROW STROMAL CELLS IN VITRO

The occurrence of a default osteogenic response in the bone marrow during establishment of local cancer metastases is rooted in the inherent osteogenic properties of the bone marrow stromal cells. The adherent (stromal) fraction of cell suspensions obtained from the post-natal bone marrow comprise a population of clonogenic cells of fibroblastic habit (colony forming unit-fibroblastic, CFU-F) in which multi-potential cells with self-renewal abilities (marrow stromal stem cells, mesenchymal stem cells, skeletal stem cells) are found (15, 16).

In vitro cultures of stromal progenitor cells are established by seeding single marrow cell suspensions at clonal density. Rapid adherence to the substrate and clonogenic ability are critical in this approach to select the non-hematopoietic, non-endothelial fraction of the stromal population, which includes osteogenic progenitors of different ranking. However, the most commonly used approaches to the *in vitro* modeling of cancer-stroma interaction have involved the use of total stromal cell cultures, which include

significant numbers of macrophages and endothelial cells. Advantages and disadvantages are attached to this approach, namely the creation of a composite *in vitro* cellular environment to some extent reminiscent of the *in vivo* situation, and the inability to model the direct interaction of the cancer cell with the stromal cell proper.

Instrumental in the ability to isolate bone marrow stromal cells directly from marrow by FACS has been the development and characterization of the mouse monoclonal antibody, Stro-1 (17, 18). The population of Stro-1 positive cells isolated from single cell suspensions of bone marrow effectively isolates virtually all of the CFU-F that can be enumerated by using the classic "colony forming efficiency" assay developed by Friedenstein (19). This assay relies on the ability of a stromal cell to become quickly adherent (unlike most hematopoietic cells, murine macrophages being the exception), and to proliferate to form a colony. However, approximately 1% of marrow cells are Stro-1 positive, a proportion far higher than the frequency of CFU-F calculated from adherent assays (~10-20 cells per 10^5 marrow mononuclear cells). Closer examination reveals that the Stro-1 population can be further subdivided based on intensity. The majority of the cells are Stro-1^{dull} and are primarily erythroblasts, whereas Stro-1^{bright} cells include all of the adherent clonogenic cells, yet not all Stro-1^{bright} cells are clonogenic (20). Stro-1^{bright} populations co-express a variety of integrins, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, while only a proportion of stromal precursors expressed the integrin $\alpha_4\beta_1$, and no measurable levels of the integrin $\alpha_3\beta_1$ were detected (21).

It was subsequently found that the Stro-1^{bright} population could be further purified by dual-color cell sorting using Stro-1 and VACM-1 (CD106) (20), and others have used similar approaches using other surface markers. The Stro1^{bright}/VCAM-1 population was negative for the hematopoietic marker, CD34. Of note, 90% of these cells expressed type I collagen, whereas 70% expressed alpha-smooth muscle actin. The dual sorted population was uniformly negative for the osteogenic transcription factors (CBFA1 and Osterix) markers of mature osteoblastic cells (osteopontin and osteocalcin), and chrondrocytes (type II collagen and aggrecan), but interestingly, routinely expressed type X collagen, a hypertrophic chondrocyte marker. In evaluation of markers of adipogenic commitment and differentiation, only LDL was expressed.

Explantation of bone marrow stromal cells *in vitro*, even those "purified" by FACS, reveals the inherent heterogeneity of the population. When seeded at high density and analyzed en mass, they are uniformly negative for hematopoietic markers. Some cells within the population display some, characteristics of endothelial cells such as CD146 (MUC-18, Mel-CAM), and endoglin, but notably are negative for Factor VIII (22). Virtually all

express type I collagen, but are variably positive for other connective tissue and osteogenic markers such as osteonectin, alkaline phosphatase, osteopontin, bone sialoprotein and osteocalcin. The same variability is noted in expression of adipogenic markers such as CEBP-alpha, PPAR-gamma2, and leptin. Interestingly, and in keeping with their potential origin as perivascular cells, some express smooth muscle markers such as alpha-smooth muscle actin and the pericyte marker, 3G5 (9,23). When plated at low density, the heterogeneity is even more pronounced in the colonies that are formed by individual CFU-Fs. Cells with different morphologies such as a large and flat cells, spindle-shaped fibroblastic cells, and a range of morphologies in between these two extremes develop colonies that grow at different rates and with different habits. In some cases, the cells are highly migratory and remain in monolayer, while others form multi-layers and display evidence of osteoblastic differentiation (24). Yet, extensive analysis of morphology, histochemical markers, patterns of gene expression and in vivo transplantation of such clonally derived strains has failed to determine the "fingerprint" by which a colony can be determined to be multi-potential *a priori* (25).

6. STROMAL CELL-CANCER CELL INTERACTIONS

There are several possible mechanisms by which bone marrow stroma mediates cancer metastasis to bone involving chemotaxis, cell-matrix, cell-cell and cell-factor interactions. It has been proposed that chemoattractant factors emanate from the marrow as a result of bone turnover, such as type I collagen degradation fragments, or products of stromal cells themselves Stromal derived factor-1, and growth factors such as TGF-beta, IGF-1 and 2, and osteonectin, attract cancer cells in the blood stream to adhere to the microvasculature and extravasate (26). Once in the extravascular spaces, cancer cells encounter a distinctly unusual extraceullar matrix that is composed of thin reticular fibers of types I, III and V collagens, presumably oriented by chondroitin and heparin sulfate proteoglycans, fibronectin and thrombospondin, and held within a gel-like substance composed primarily of hyaluronic acid. Although there are a number of studies describing the interaction of cancer cells with various different matrix components *in vitro*, the interactions of cancer cells with matrix as it exists *in situ* remain to be characterized.

Direct cell-cell contact undoubtedly mediates a number of events in the establishment of a metastasis, although not well defined to date. It is possible that molecules that govern stromal cell-hematopoietic cell interactions

[integrins, selectins, the immunoglobulin superfamily, and mucin-like molecules, reviewed in (27)] may also be operative in stromal-cancer cell interactions. Based on the expression of CD44, a hyaluronic acid receptor, by both hematopoietic progenitor cells and myeloma cells, it had been assumed that tumor cells (in particular, with variant forms of CD44) interact with hyaluronic acid in the extravascular spaces. More recent findings suggest that the receptor binds directly to an as yet unidentified ligand on stromal cells (28), which in turn stimulates the production of IL-6 by stromal cells (29).

Finally, it is clear that cell-factor interactions have a profound effect on progression of metastatic bone disease. Metastatic cells in the marrow microenvironment are bathed in a rich milieu of growth factors and cytokines including (but not limited to) bFGF, IGFs, PDGF, EFG, TGF-beta and BMPs that are capable of stimulating cancer cell growth, as in prostate cancer. (30, 31). Furthermore, these stromal-derived factors induce cancer cell expression of factors such as PTHrP, IL-1, IL-6, IL-11 and TNF-alpha and beta (32), which are known to stimulate the formation and activity of osteoclasts. PTHrP, in particular, has been implicated in stromal cell expression of RANKL, an essential component for initiation of osteoclast formation (33). Furthermore, many of these cytokines induce a variety of proteinases in stromal cells and cancer cells alike, although a full description of the patterns of induction is not available. Thus, the interplay between stromal cells and cancer cells results in the establishment of an osteolytic process, characteristic of end stages of most types of metastatic bone disease (34).

7. STROMAL CELLS AND THE MODELING OF BONE METASTASIS

Either multi-colony-derived strains or some single colony-derived (clonal) strains of *ex-vivo* expanded marrow stromal cells are able to generate, when transplanted in the subcutaneous tissue of immunocompromised mice, a host of connective tissues of donor origin, including bone, adipose tissue, fibrous tissue, a reticular-fibroblastic stroma capable of supporting hematopoiesis, and also, under specific experimental conditions, cartilage. These tissues are organized in an orderly structure, which overall, faithfully recapitulates the structure of any anatomical site with a trabecular bone/bone marrow structure. Furthermore, the marrow spaces formed among the bony trabeculae become colonized by a complete hematopoietic tissue, in which all major lineages undergo a normal differentiation/maturation sequence. Experiments using markers of species

origin of the different tissues (e.g., *in situ* hybridization experiments employing probes specific for human specific Alu sequences) have demonstrated that while the bony structures and the associated stromal tissues are of donor origin, the hematopoietic cells colonizing the ectopic miniature of a skeletal site (also called the "ectopic ossicle") are of host origin (15,19). Thus, the transplanted cells generate not only bone, but also a hematopoietic microenvironment, which then becomes seeded by blood-borne murine hematopoietic stem cells, which in turn generate a hematopoietic tissue (35). In these systems, thus, the classical "seed-and-soil" paradigm underlying the normal ontogeny of the bone/bone marrow organ [and also, as recognized by Stephen Paget as early as 1889, for the ontogeny of bone metastasis, (1)] is reproduced, and the ultimate outcome of the procedure essentially represents a mirror image of the chimeric bone marrow generated by transplantation of hematopoietic progenitors also in humans (where the hematopoietic cells are of donor origin, and the stroma is of host origin) – indeed, a true "reverse" bone marrow transplantation (36).

Multi-potent progenitors capable of self-renewal and of generation of both bone tissue and a hematopoietic microenvironment represent bona fide skeletal stem cells, whereas cells capable of more restricted differentiation capacity, limited to the ability of generating bone tissue, but not bone marrow stroma, upon transplantation, are regarded as committed osteogenic progenitors (4). Both types of progenitors may participate in the osteogenic response to cancer metastasis. However, short-lived, default osteogenesis occurring in virtually all metastasis, and sustained osteosclerosis restricted to specific types of bone metastasis may involve the recruitment and activation of distinct osteogenic progenitors in the bone marrow. This, in turn, may be rooted into the specific biological activity of cancer cells.

In vivo transplantation assay forms the basic experimental approach to the investigation of the differentiation and organogenic potential of skeletal stem cells under normal conditions, and of their use on a larger scale for tissue engineering and cell therapy of skeletal tissues (37). We have shown that these systems can also be bent to the investigation of genetic diseases of the skeleton, by transplanting strains of human skeletal progenitor cells derived from the bone marrow of patients carrying specific genetic anomalies. In this type of application, mutated human skeletal progenitor cells generate miniature replicas of specific human skeletal diseases of known genotype (38,39), or of unknown genotype (40). Likewise, transplanted skeletal stem cells effectively demonstrate the cell-autonomous skeletal effects of targeted mutations of murine genes (41). Thus, these systems have a specific value in investigating human diseases and in generating advanced models thereof.

This system can provide a valuable model for investigating mechanisms of the homing of cancer cells to the human bone/bone marrow environment, an essential step in the development of bone metastasis. The very fact that the ectopic skeletal tissues become colonized by murine hematopoietic cells proves that blood-borne (hematopoietic) cells capable of recognizing the specific bone microenvironment can be home to the ectopic environment with similar efficiency as to the orthotopic bone. Thus, one can envision that cancer cells inoculated intravenously in animals bearing an ectopic ossicle would seek the ectopic human bone marrow microenvironment, seed it, and develop into an ectopic mini-metastasis in human bone. The resulting model would have two significant fallouts. The availability of human bone and marrow as homing sites would permit modeling of metastasis of tumor types that do not efficiently seed mouse bone, such as prostate cancer. Thus, a model of prostate cancer metastasis for conventional studies including those aiming at bone changes (e.g., induction of osteoclastic bone resorption, or stimulation of excess bone formation) or drug testing would become available. The other and more significant one derives from the fact that the bone environment itself, in the envisioned system, is built *de novo*, *in vivo*, with cells previously isolated and expanded in culture. This means that the system allows for the manipulation of both partners of the cancer cell/stromal cell interaction, thus setting the stage for an *in vivo* experimental approach to the molecular mechanisms of their interaction.

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Chapter 6

THE ROLE OF TUMOR-ASSOCIATED MACROPHAGES IN METASTASIS-ASSOCIATED OSTEOLYSIS

Nicholas A. Athanasou
Departments of Surgery and Pathology, Nuffield Department of Orthopaedic Surgery, University of Oxford, Nuffield Orthopaedic Centre, Oxford, UK*

1. INTRODUCTION

Solid tumors frequently metastasise to bone and bone metastases often occur in patients with common cancers such as breast, prostate and lung; these tumors not only have the potential for metastasis at a relatively early stage of the disease but can also produce metastases many years after adequate local treatment (1, 2). A significant macrophage infiltrate is often found in these metastatic cancers. On the basis that monocytes and macrophages are able to recognise and kill malignant cells *in vitro* and that most human tumors are infiltrated by macrophages, cells of the mononuclear phagocyte lineage have long been considered to play a role in the host response to tumors (3, 4). Macrophages have also been implicated in the pathogenesis of bone metastases, although the precise role that these cells play in the growth and development of secondary tumors in bone is not established. One means whereby tumor-associated macrophages (TAMs) may affect the growth of metastatic tumors in bone is through influencing the formation and activity of osteoclasts; multinucleated cells which are specialized to carry out bone resorption.

This chapter reviews the pathobiology of macrophages, particularly TAMs and the role that these cells play in tumor osteolysis. This chapter includes a discussion of the cellular and humoral mechanisms whereby

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monocytes and macrophages have been shown to differentiate into osteoclasts, and the relationship of these mechanisms to tumor-associated osteolysis.

2. MACROPHAGE BIOLOGY

The mononuclear phagocyte system (MPS) consists of a family of closely related cells of bone marrow origin including blood monocytes, tissue macrophages and tissue-specific cells such as Kupffer cells in the liver, Langerhans cells in the skin, microglia in the central nervous system and osteoclasts in bone (5). Macrophages are mononuclear inflammatory cells which are found scattered in connective and parenchymal tissues of the body. They are commonly seen as part of the inflammatory response to neoplastic and non-neoplastic conditions. The single most important characteristic of macrophages and other cells of the MPS is their ability to carry out phagocytosis avidly and efficiently (6).

All cells of the MPS arise from the pluripotential haematopoietic stem cell from which other circulating cells are derived (6, 7). The proliferation and differentiation of haematopoietic cells is controlled by a series of growth factors exhibiting varying degrees of lineage specificity. There is considerable evidence to suggest that monocytes and granulocytes share a common colony-forming unit granulocyte-macrophage (CFU-GM) progenitor cell. After this progenitor cell becomes committed to monocyte or granulocyte differentiation, monoblasts and promonocytes are formed in the bone marrow, and finally monocytes are released into the circulation. Newly formed monocytes remain in the bone marrow for less than 24 hours before entering the bloodstream where they are distributed between circulating and marginating populations (6, 8). Monocytes subsequently migrate into various tissues and transform into macrophages. The half-life of blood monocytes is about one day, whereas the life span of tissue macrophages is several months. Although most tissue macrophages are derived from monocytes, there is evidence that up to 5% of macrophages are derived from the local division of mononuclear phagocytes in the tissues (8, 9). Macrophages in tissues and body cavities do not represent a constant population of cells but are regularly being renewed by the influx of new monocytes.

In the presence of an appropriate environmental stimulus, macrophages are capable of differentiating into cells with altered functional properties (4, 6). Macrophages can be activated by cytokines secreted by sensitized T lymphocytes, bacterial endotoxins, other chemical mediators and extracellular matrix proteins such as fibronectin (10). Following activation, macrophages increase in cell size, produce an increased level of lysozyme

and other enzymes, and act as important mediators of tissue destruction, vascular proliferation and fibrosis. These are all features characteristic of chronic inflammation as well as the host response to tumor growth. Receptors on the surface of macrophages include those for the Fc region of the IgG molecule and the cleavage product of the third component of complement (C3) (6, 11, 12). Macrophages also possess receptors for cytokines, growth factors and hormones and express a number of characteristic surface antigens, some of which are relatively lineage-specific, such as CD14 and CD68 (13, 4). Mononuclear phagocytes also express lymphocyte function-associated (LFA) antigens, one of the integrin family of cell adhesion molecules (15, 16). Inflammatory mediators such as lipopolysaccharide, interferon γ (IFNγ), interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) cause strong induction of ICAM-1 (CD54) in a wide variety of tissues and greatly increase the binding of monocytes through cell surface LFA-1 (CD11a/CD18) (16). This contributes to the infiltration of mononuclear phagocytes into sites of pathology. Two other integrins, Mac-1 (CD11b/CD18) and p150, 95 (CD11c/CD18) are also important in adhesion of mononuclear phagocytes to ligands which become insoluble during activation of complement and the clotting cascade and in binding to other cells. A number of the above macrophage-associated antigens are not expressed by specialized tissue-specific MPS cells such as Langerhans cells and osteoclasts (17, 18).

3. TUMOR-ASSOCIATED MACROPHAGES (TAMS)

Most benign and malignant tumors are composed of two distinct cellular components, neoplastic cells and non-neoplastic cells. The latter includes connective tissue elements, blood vessels and inflammatory cells. The inflammatory cell infiltrate is composed mainly of lymphocytes and macrophages and is thought to represent evidence of a host response to the tumor (4, 19). TAMs form a major component of the inflammatory cell infiltrate within and around primary and metastatic tumors (20, 21). TAM numbers infiltrating a tumor depend on the stage of tumor development (22, 23). A relatively high macrophage: tumor cell ratio is seen early in tumor development but with increasing tumor growth, the inflammatory response to the tumor is less pronounced and the macrophage content of tumors is consequently reduced. At sites of metastasis, TAM numbers are highest soon after secondary implantation (21); in bone, this is when malignant bone resorption is proceeding most rapidly and osteoclasts and osteoclastic resorption is prominent within skeletal metastases (Figure 1) (see below).

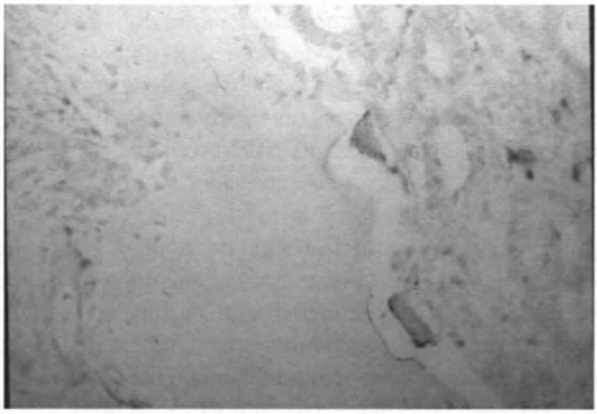

Figure 1. Photomicrograph of a metastatic breast cancer in bone stained immunohistochemically for the pan-mononuclear phagocyte marker CD68, which reacts with TAMs in the tumor deposit and osteoclasts on the bone surface undergoing resorption (Immunoperoxidase X100).

Tumors are known to release several factors which induce chemotactic migration of monocytes such as monocyte chemotactic protein-1 (MCP-1) (3, 24). Although MCP-1 does not directly influence osteoclast formation, it is produced by cancer cells and human osteoblastic cells, and could regulate recruitment of mononuclear phagocyte osteoclast precursors into sites if bone metastases (24, 25, 26). Pro-inflammatory cytokines, including IL-1β, TNFα, transforming growth factor-β (TGF-β) and interleukin-6 (IL-6), which are produced by osteoblasts and tumor cells, stimulate MCP-1 production. Other factors which promote macrophage infiltration into tumors include macrophage colony stimulating factor (M-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF) and RANTES (3, 4). Proliferation and survival of TAMs has been related to tumor cell production of M-CSF and TAM expression of the c-fms proto-oncogene which encodes the M-CSF receptor, (27). TAMs secrete various other mediators that influence tumor cell growth and regulation (progression and regression), angiogenesis, fibrin deposition, invasion and metastasis, local and systemic immunosuppression and production of acute phase proteins (3, 4).

4. TAMS AND OSTEOLYTIC METASTASES

A role for TAMs in causing tumor osteolysis was first suggested by the histopathology of metastatic lesions in bone. Histological examination of

bone biopsies from the vertebrae of sixty patients dying from various malignant tumors and of lesions produced by injection of VX2 carcinomas directly into the tibia or periosteum of rats appeared to show that tumor osteolysis associated with metastatic cancer occurred in two somewhat distinct phases that consist of: (i) an early phase when the tumor cells are becoming established and bone resorption proceeds rapidly, at which time osteoclasts and resorption lacunae are very prominent, and (ii) a later phase when the metastasis continues to enlarge but at a slower rate, at which time tumor cells and tumor-associated inflammatory cells lying next to residual bone spicules dominate the histological picture and osteoclasts are not numerous and in many cases absent (28, 9). Histological studies of the direct invasion of bone by head and neck cancers showed that the cellular response accompanying osteolysis was similar to the two stage process described above for osteolytic metastases and noted that tumor associated inflammatory cells, particularly TAMs, might play a role in directly resorbing bone (30, 1). Hulth and Olerud, however, using the VX2 carcinoma model, concluded that osteoclasts did not appear to contribute significantly to tumor osteolysis. Most of the malignant bone resorption apparently being carried out by the tumor cells themselves cells within the implanted VX tumors (32).

5. CELLULAR FACTORS CONTROLLING MONONUCLEAR PHAGOCYTE DIFFERENTIATION TO OSTEOCLASTS

Osteoclasts are multinucleated cells that are specialized to carry out lacunar bone resorption. Osteoclasts are efficient phagocytes and form part of the MPS (5, 33, 34). Osteoclasts are not commonly seen in normal adult bone but are present at sites of bone resorption associated with most diseases that affect bones and joints. Osteoclasts were originally thought to share a common progenitor cell with bone-forming osteoblast cells, but it is now well-established that osteoclasts are of haematopoietic rather than bone stromal cell origin (35). Mononuclear osteoclast precursors are products of the pluripotential haematopoietic stem cell from which other marrow elements and circulating leucocytes are derived. The osteoclast lineage shares marrow precursors with monocytes and macrophages including committed precursors e.g., colony forming unit-granulocyte macrophage (CFU-GM) for cells of this lineage (36). These marrow-derived mononuclear osteoclast precursors are released into the circulation and fuse to form osteoclasts at the bone surface.

The precise osteoclast lineage pathway was not fully determined until the cellular and molecular factors that control this process were discovered. One particular area of controversy concerned the relationship of osteoclasts to monocytes and tissue macrophages. Resolution of this controversy required the establishment of experimental systems whereby osteoclasts could be induced to differentiate *in vitro* from marrow and circulating precursors. In long-term mouse marrow culture systems, it was shown that osteoclast-like cells formed from marrow precursors and that osteotropic hormonal factors, such as 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), parathyroid hormone (PTH) and prostaglandin E_2 (PGE2) can influence this process (37). The importance of bone stromal cells and M-CSF for osteoclast formation was subsequently shown in cocultures of mouse and human marrow stromal cells and haematopoietic precursors (38). It was later found in rodents that osteoclasts can be formed in cocultures of bone stromal cells and mature monocytes or macrophages (39, 40). Using a similar coculture system, it was shown that the human osteoclast precursor circulates in the monocyte fraction and that osteoclasts form in cocultures of human monocytes and osteoblast-like cells in the presence of $1,25(OH)_2D_3$ and human M-CSF (41). Human tissue macrophages isolated from various normal and pathological tissues were shown to be similarly capable of differentiating into osteoclasts when cocultured with osteoblasts under these conditions (42, 43, 44). M-CSF is an absolute requirement for the proliferation and differentiation of osteoclast precursors (45). M-CSF acts through c-fms, the M-CSF receptor and its signal transduction is mediated by tyrosine kinase (27).

The human mononuclear osteoclast precursor expresses a macrophage phenotype, including cell surface makers being $CD11^+$ and $CD14^+$ is incapable of carrying out lacunar resorption, and is negative for phenotypic markers of the mature osteoclast phenotype, such as tartrate-resistant acid phosphatase (TRAP), the vitronectin receptor (VNR) and calcitonin receptor (41). Only a minority of circulating mononuclear cells (approximately 2-5%) within the human monocyte fraction appear to be capable of osteoclast differentiation. Osteoclast differentiation from circulating precursors in the $CD14^+$ monocyte fraction involves stepwise loss and acquisition of specific phenotypic markers for macrophages and osteoclasts respectively (46, 47).

6. MOLECULAR FACTORS CONTROLLING MONONUCLEAR PHAGOCYTE DIFFERENTIATION TO OSTEOCLASTS

As direct cell-cell interaction between mononuclear phagocyte osteoclast progenitors and bone stromal cells appeared to be essential for osteoclastogenesis, it was postulated that a membrane-associated factor was present on bone stromal cells and that this ligand interacted with a receptor on osteoclast precursors. This factor was discovered by expression cloning of a protein that binds osteoprotegerin (OPG), a protein which was found to block osteoclast development *in vitro* and *in vivo* (48, 49). OPG-deficient mice exhibit severe osteoporosis and transgenic mice over-expressing OPG develop severe osteopetrosis (49, 50). The ligand to which OPG binds is a polypeptide of 317 amino acids that belongs to the TNF-ligand family. It had previously been identified as TNF-related activation inducing cytokine (TRANCE), a molecule which activates the receptor activator of nuclear factor κB (RANK) (51, 52). When it was shown that soluble forms of RANKL (in the presence of M-CSF) induce osteoclast formation from spleen cells and monocytes in the absence of bone cells, it was clear that RANKL represented the osteoclastogenic factor that is expressed by bone stromal cells (53).

Although RANKL exists in two forms, a membrane-bound form (40-45kD) and a soluble form (31kD) it is mostly cell bound and produced by activated T lymphocytes and osteoblasts/bone stromal cells (54, 55, 56). RANKL mRNA are most abundant in skeletal tissues and lymphoid tissues that are active in mediating an immune response (e.g., lymph node, thymus, spleen, Peyer's patches) (54, 57). RANKL has 20-35% homology with the TNF-related apoptosis inducing ligand (TRAIL) and OPG is known to bind to TRAIL (54, 56). The human RANKL gene is located on chromosome 13 (13q14). A response element for Cbfa-1, the transcription factor which plays a key role in osteoblast differentiation, has been identified on the promoter of the mouse RANKL gene. It is also of significance that levels of RANKL mRNA are increased by many hormonal and humoral factors which promote bone resorption, including parathyroid hormone (PTH), glucocorticoids, $1,25(OH)_2D_3$, TNFα, IL-1β and PGE2. RANKL mRNA expression are suppressed by TGF-β (54, 56).

RANKL interacts with RANK, a type I transmembrane protein of 616 amino acids that is a member of the TNF receptor superfamily. The human RANK gene is encoded on chromosome 18 (18q22.1) (54, 56). RANK is a member of the TNF receptor superfamily and is a type I transmembrane protein which, in bone, is mainly expressed by cells of the osteoclast lineage. Although RANK mRNA expression has been found in a variety of tissues,

the protein form of RANK is found mainly on osteoclasts, monocytes, macrophages, dendritic cells, B and T lymphocytes and fibroblasts. RANK expression by T cells is stimulated by IL-4 and TGFβ. RANKL-RANK signalling results in activation of the transcriptional factor NF-κB; this process involves interaction with several TNF-receptor-associated factors (TRAFs), including TRAFs 2, 5, and 6. RANK, NF-κB and TRAF-6 knock out mice are all osteopetrotic.

The RANKL-RANK interaction plays a key role in osteoclast formation and resorption. RANKL stimulates osteoclast formation, promotes the resorbing activity of mature osteoclasts and inhibits the apoptosis of these multinucleated cells (52, 53, 54). Stimulation of osteoclast formation by RANKL has been shown in both cultures of haematopoietic cells and in human peripheral blood ($CD14^+$) monocyte and macrophage populations (58, 59). Stimulation of osteoclast formation by RANKL is dose-dependent and is specifically inhibited by OPG. Transgenic mice that overexpress RANKL exhibit severe hypercalcaemia and osteopaenia associated with an increase in the osteoclast resorption and lacunar resorption. In contrast, RANKL knockout mice exhibit severe osteopetrosis and defects in tooth eruption associated with an absence/deficiency of osteoclasts; these mice also lack lymph nodes and show defects in thymic differentiation as well as abnormal mammary gland development (54, 55, 56).

OPG is a secreted 110kD soluble TNF decoy receptor for RANK that competitively inhibits the RANKL-RANK interaction (50, 54). The human OPG gene is on chromosome 8 (8q23-34). It is secreted as a soluble protein of 401 amino acids which is then cleaved to the active peptide. In contrast to RANKL and RANK, OPG has a very wide tissue distribution. In addition to bone and the immune system, it is found in the lung, heart, kidney, liver, stomach, intestine, skin, brain and central nervous system. In bone, OPG is produced by osteoblasts and bone marrow stromal cells. OPG mRNA has also been found in endothelial cells, aortic smooth muscle cells, fibroblasts as well as some cancer, monocyte, dendritic and B lymphocyte cell lines (54, 55, 56). Levels of OPG mRNA and protein in osteoblastic cells are increased by oestrogen, PTH, $1,25(OH)_2D_3$, IL-1, TNFα, TNF-β and BMP2; OPG mRNA and protein levels are decreased by glucocorticoids and prostaglandins. OPG inhibits the final stages of osteoclast formation and, at high concentrations, inhibits mature osteoclast activity and induces osteoclast apoptosis. OPG also acts as a soluble receptor for TRAIL and inhibits TRAIL-induced apoptosis. OPG knock out mice have severe osteoporosis and exhibit extensive bone resorption; they also exhibit marked vascular calcification localized to the media of large arteries (55).

7. OSTEOCLAST FORMATION AND TUMOR OSTEOLYSIS

Identification of the cellular and humoral conditions under which marrow-derived circulating mononuclear phagocyte osteoclast mononuclear precursors are capable of differentiating into mature bone resorbing osteoclasts has clear implications for pathological conditions, such as metastatic bone disease, where there is significant osteolysis. It was first shown that TAMs, isolated from primary mammary carcinomas in C3H/Avy mice, which express a macrophage and not osteoclastic phenotype (ie non-specific esterase+ TRAP-, F4/80+, and unresponsive to calcitonin), were capable of differentiation into TRAP+ osteoclastic cells capable of extensive lacunar bone resorption upon coculture with bone derived stromal cells in the presence of $1,25(OH)_2D_3$ (59). It was also shown that human TAMs (TRAP-, CD14+, calcitonin unresponsive) isolated from primary lung and breast carcinomas could also exhibit osteoclastic differentiation (60). These early studies showed that TAMs could contribute to malignant bone resorption by undergoing differentiation into osteoclasts.

As with all other mononuclear phagocyte populations cultured in this way, a close correlation between the appearance of TRAP positivity and the onset of bone resorption was seen in TAM-bone stromal cell cocultures. The formation of colony-like clusters of TRAP+ cells in these cocultures and the necessity for prolonged (i.e. greater than 3 days) coculture of mononuclear phagocytes with stromal cells in the presence of $1,25(OH)_2D_3$ before the appearance of TRAP+ multinucleated cells and the commencement of bone resorption, indicated that the action of the hormonal and bone stromal cell elements was to favour differentiation of a subpopulation of TAMs into osteoclastic bone-resorbing cells. This most likely occurs by promoting expression of RANKL (and/or RANK) in these cultures. It has been shown that macrophages isolated from breast cancers and malignant mesenchymal tumors are capable of osteoclast differentiation when these cells are cultured in the presence of M-CSF and RANKL and that this process is inhibited by osteoprotegerin and RANK:Fc, a soluble form of RANK (Figure 2) (61,62).

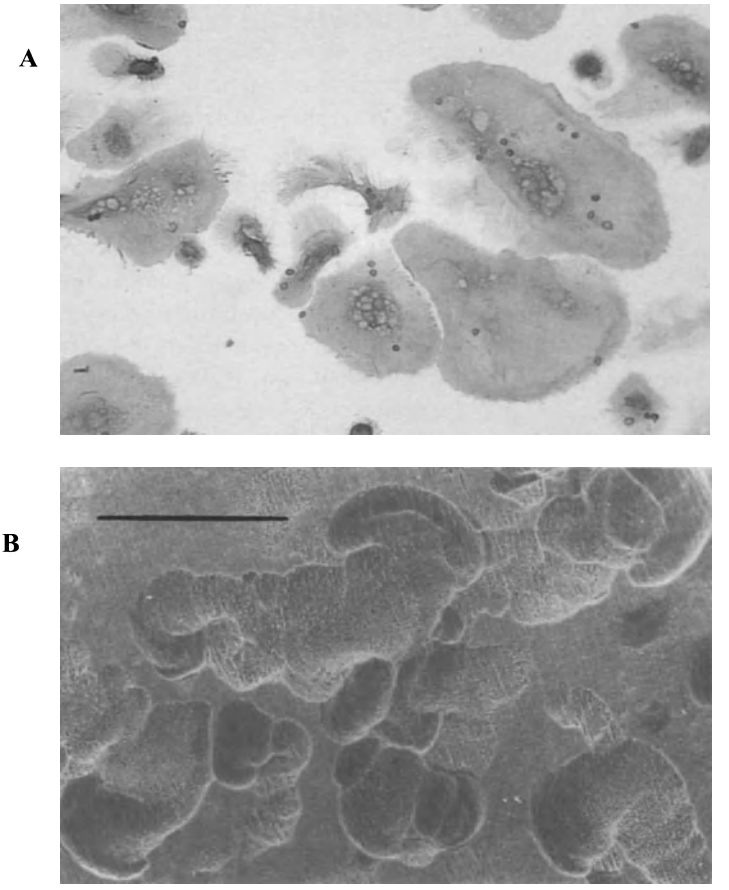

Figure 2. Osteoclast formation from CD14+ TAMs cultured with soluble RANKL and M-CSF is evidenced by the formation of multinucleated cells that are:
a) Vitronectin receptor (CD51)- positive (Immunoperoxidase X200)
b) Capable of lacunar resorption pit formation (Bar = 100μ).

To determine whether the increase in osteoclast numbers in skeletal metastasis is associated with an increase in circulating osteoclast precursors or increased sensitivity of these precursors to humoral factors required for osteoclast formation, monocytes were isolated and cocultured with RANKL-positive osteoblastic cells in the presence of $1,25(OH)_2D_3$ and M-CSF (63). Limiting dilution experiments showed that there was no increase in the number of circulating osteoclast precursors in breast cancer patients with

skeletal metastases (± hypercalcaemia) compared to normal controls or breast cancer patients without have bone metastases. In addition, circulating osteoclast precursors in these patients were not hyper-responsive to $1,25(OH)_2D_3$ or M-CSF, factors which are absolute requirements for osteoclast formation in this coculture system. These results indicated that the increase in osteoclast formation in breast cancer skeletal metastases is not due to an increase in the number or nature of circulating osteoclast precursors.

It was also found that the conditioned medium from cultured breast cancer cell lines, (MCF-7 and MDA-MB-231), stimulates osteoclast formation and resorption when monocytes are cultured with RANKL-expressing osteoblastic cells (63). These results suggest that tumor cells promote osteoclast formation in the bone microenvironment by producing soluble osteoclastogenic factors. The nature of these soluble factors is uncertain as circulating osteoclast precursors isolated from breast cancer patients did not exhibit increased sensitivity to $1,25(OH)_2D_3$, M-CSF, PTHrP and IL-6 in terms of osteoclast formation relative to normal controls. Increased numbers of $TRAP^+$ cells has also been noted in human bone marrow cultures exposed to the conditioned medium of cultured breast cancer cells (64). Tumor cells are known to secrete factors, some of which (e.g., IL-3 and GM-CSF) promote osteoclast formation from monocyte precursors. Lee et al. showed that an osteoclast CSF is released in cultures of a cell line derived from a murine mammary carcinoma that induces hypercalcaemia (65). Other cultured breast cancer cell lines, known to produce prostaglandins and leukaemia inhibitory factor, have also been shown to promote osteoclast formation by a stromal cell-dependent pathway (66, 67).

A number of studies which have provided evidence that the RANKL/RANK/OPG cytokine system may play a role in the production of osteolytic metastases and the humoral hypercalcaemia of malignancy in breast cancer are also likely to relevant to TAM-osteoclast differentiation. It has been shown that the breast carcinoma cell lines MDA-MB-231, MCF-7 and T47D as well as tumor cells isolated directly from primary breast cancers do not express RANKL but do express OPG and RANK (68). These cancer cells cannot replace RANKL-expressing osteoblastic cells which support osteoclast formation in coculture experiments with mouse marrow cells. However, osteoclast formation is stimulated when MCF-7 cells which over-express PTHrP are added to cocultures of mouse osteoblasts and haematopoietic cells. These MCF-7 cells enhance RANKL mRNA and inhibit OPG mRNA production by bone stromal cells. It has also been shown that breast carcinoma cells secrete high levels of PTHrP and that this enhances RANKL and inhibits OPG mRNA production by osteoblastic cells

(69). This increase in the RANKL: OPG ratio induced via PTHrP production by breast carcinoma cells was associated with stimulation of osteoclast differentiation and resorption. Kitazawa et al. also found that RANKL expression was confined to bone stromal/osteoblastic cells when PTHrP-producing breast cancer cells were injected into mice (70). They concluded that PTHrP induces osteoclastic bone resorption through the transactivation of the RANKL gene on stromal/osteoblastic cells, in this way providing a bone microenvironment which is favorable to the survival of PTHrP-producing cancer cells. Thus, in the osteolysis of skeletal metastasis in breast cancer, tumor cells which do not express RANKL, may induce local over-expression of RANKL in osteoblasts leading to increased osteoclast formation and resorption.

In keeping with increased osteoclast formation from circulating or marrow precursors as one of the mechanisms of malignant bone resorption, it has been shown that OPG inhibits the development of osteolytic carcinomatous metastases and the extent of osteolysis in bone metastases that are produced following injection of human breast cancer cells into nude mice (71). These breast cancer cells have also been shown to exhibit constitutive expression of M-CSF mRNA and to increase osteoclast formation by secreting M-CSF and upregulating RANKL in bone marrow stromal cells (72). TGF-β, which is abundant in the bone matrix and released as a consequence of osteoclastic resorption, may play a key role in tumor osteolysis. TGFβ appears to stimulate PTHrP secretion from breast cancer cells by a post transcriptional mechanism through both SMAD and P38 mitogen activated protein (MAP) kinase signalling pathways (73, 74). Osteolytic metastases can be suppressed by a blockade of TGF-β signalling in tumors and by neutralization of PTHrP, suggesting that a vicious circle involving TGFβ release from resorbed bone may enhance tumor cell growth and stimulation of osteoclast formation via PTHrP (73, 74, 75).

There is conflicting evidence as to whether RANKL is expressed by carcinoma cells in metastatic bone lesions. Huang et al. examined cases of breast, lung, prostate and thyroid cancer which had metastasized to bone and noted expression of RANKL mRNA and protein by in situ hybridization and immunohistochemistry (76). They concluded that metastatic carcinoma cells, regardless of origin, express RANKL and that these cells may directly stimulate osteoclast formation and resorption. RANKL expression has also been noted in primary and secondary osteolytic human bone tumors and myeloma (77). Although most RANKL is membrane-bound it is possible that tumor cells could produce a soluble form of RANKL. A squamous cell carcinoma cell line derived from a primary tumor which causes severe hypercalcaemia has been found to produce soluble RANKL (78). Whether RANKL is membrane-bound or secreted, it is likely that it plays a role in

osteoclast formation from TAMs (Figure 3). It has been shown that monocytes, inflammatory macrophages and TAMs are capable of osteoclast formation in the presence of M-CSF and membrane-bound or soluble RANKL (42, 44, 59, 62).

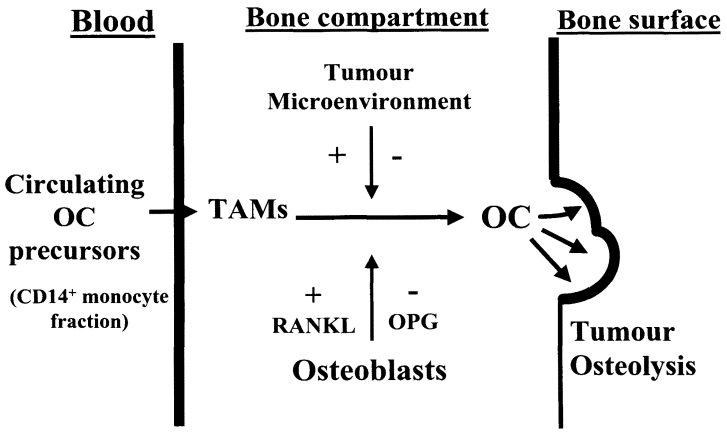

Figure 3. Scheme of RANKL-induced TAM-osteoclast (OC) differentiation and tumor osteolysis. CD14+ precursors (monocytes and TAMs) express RANK and interact with RANKL on osteoblasts in the presence of M-CSF to differentiate into osteoclasts. Humoral factors produced by neoplastic and non-neoplastic cells in the tumor microenvironment promote (e.g. M-CSF, PTHrP, TGF-β) or inhibit (e.g. OPG) this process. RANKL, OPG and humoral factors also influence osteoclast activity and survival.

Zhang et al. showed that prostate cancer cell lines produce a soluble form of RANKL which stimulates osteoclast formation (79). Injection of these cancer cells into bone resulted in the formation of an osteolytic tumor but the establishment of this tumor was prevented by OPG treatment. Their results provide strong evidence that tumor cells can stimulate tumor osteolysis by osteoclast production and secretion of RANKL. Whether prostate cancer cells similarly influence osteoclast differentiation from TAMs has not been tested. Prostate cancer cells have also been shown to produce OPG and to release biologically significant quantities of this molecule (80). This acts as a survival factor for prostate cancer cells by binding to TRAIL, thus preventing tumor cell apoptosis (81). This anti-apoptotic effect of OPG is reversed by RANKL. Cytokine effects on OPG production by prostate cancer cells are stimulated by TNFα and IL-1β in a dose-dependent fashion (80). Dexamethasone has a small but not significant inhibitory effect on OPG secretion from prostate cancer cell lines.

There is considerable interest in the use of OPG as a therapeutic agent to control tumor osteolysis (82, 83). OPG neutralizes RANKL and serves as a weak decoy receptor for the apoptosis-inducing TNF ligand TRAIL (53, 54). It was found that OPG treatment substantially and rapidly reduces the increased bone resorption seen in animals with hypercalcaemia induced by cytokines (e.g. RANKL, IL-1β, TNFα) or hormones (e.g. PTH, PTHrP, $1,25(OH)_2D_3$). Animal studies have also shown that rodents with tumors that produce either hypercalcaemia or skeletal metastases respond favourably to OPG (84, 85). OPG treatment reversed the established hypercalcaemia seen in a mouse model of humoral hypercalcaemia of malignancy induced by colon carcinoma (85). OPG reduced bone resorption but had no apparent effect on tumor growth, tumor-induced cachexia or PTHrP levels. OPG also inhibited the development and progression of skeletal metastases that are induced in athymic/syngeneic mice following systemic administration of colon and breast cancer carcinoma cells (71). OPG levels are not increased in cancer patients with bone secondaries and are in fact decreased in patients with myeloma and lymphoma (86); increased OPG levels are found in patients with primary pancreatic and colorectal carcinomas. OPG has been shown to decrease cancer-induced skeletal destruction and skeletal pain in a mouse model of osteosarcoma (87). RANKL-dependent osteoclast formation from mesenchymal tumor-associated macrophages can also be inhibited by OPG (62), and there may be scope for OPG or RANK: Fc treatment of other primary osteolytic tumors, such as giant cell tumor of bone and giant cell reparative granuloma of the jaw, which contain numerous osteoclasts (and osteoclast precursors) that express RANK and a substantial mononuclear osteoblast-like stromal cell component that expresses RANKL. Potential adverse affects of OPG treatment includes blockage of TRAIL which may result in inhibition of cancer cell apoptosis. Any therapeutic advantage obtained through inhibiting tumor osteolysis by OPG therefore needs to be balanced against possible enhancement of cancer cell survival and acceleration of tumor growth (81).

8. OSTEOCLAST FORMATION BY CYTOKINE INDUCED (RANKL-INDEPENDENT) MECHANISMS

A number of pro-inflammatory cytokines are known to influence RANKL-dependent osteoclast formation (54). Most of these cytokines, such as TNFα, act via receptors on osteoblasts or bone stromal cells to increase RANKL expression. TNFα dramatically increases mouse osteoclast

formation from marrow precursors by increasing the level of RANKL in bone stromal cells. On this basis it was concluded that TNFα induces osteoclast formation by directly stimulating osteoclast differentiation of marrow macrophages that are exposed to permissive levels of RANKL (88). However, It has been shown that in cultures of mouse bone marrow cells which are first incubated with M-CSF for 2 - 3 days, and subsequently with M-CSF and TNFα (± IL-1α), multinucleated cells which express the cytochemical and functional (i.e. osteolytic) characteristics of osteoclasts are formed (89, 90). TNFα acts on two cell surface receptors, Type I (TNFR1, p55) and Type II (TNFR 2, p75). Mouse TNFα binds to both TNFR-1 and TNFR-2 on mouse bone marrow macrophages. Adding antibodies against TNFR-1 and TNFR-2 blocked the osteoclast formation induced by TNFα but not by RANKL. These results indicated that TNFα induces osteoclast differentiation through TNF receptors and not RANK. Human osteoclast precursors isolated from monocyte and macrophage populations are also capable of TNFα-induced (RANKL-independent) osteoclast formation which is not inhibited by OPG or RANK: Fc (91). Other pro-inflammatory cytokines such as IL-6 and IL-11, which are increased in the inflammatory and tumor microenvironment can similarly promote osteoclast formation by a RANKL-independent mechanism (92). Osteoclast formation produced by RANKL-independent mechanisms is qualitatively different, resulting in the formation of small osteoclasts which produce correspondingly smaller than normal lacunar resorption pits (Figure 4). Glucocorticoids, which are commonly used to treat inflammatory conditions of bone and joint, such as rheumatoid arthritis, markedly inhibit TNFα-induced human osteoclast formation (91). In contrast, glucocorticoids promote RANKL-induced osteoclast formation from circulating precursors (93).

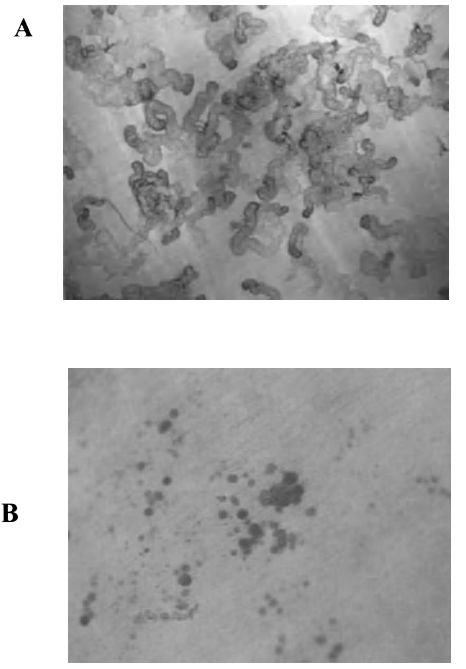

Figure 4. a) Large and b) small lacunar resorption pits produced by osteoclasts formed from monocytes by RANKL-induced and TNFα-induced (RANKL-independent) mechanisms respectively (Toluidine blue X100).

The above findings argue for two distinct pathways of osteoclast formation being present in bone, one cytokine-induced, the other RANKL-induced. These pathways may operate under different tissue conditions. At sites of physiological (e.g. normal bone turnover) and pathological (e.g. osteoporosis) bone resorption where few or no inflammatory cells are present in the skeleton, and levels of pro-inflammatory cytokines are therefore likely to be low, RANKL-induced osteoclast formation would predominate. In contrast, in inflammatory and neoplastic diseases affecting bone, where numerous inflammatory cells and pro-inflammatory cytokines are produced, TNFα, IL-6 (± IL-1α)-induced osteoclast formation would predominate (Figure 5). Anti-cytokine treatment has been shown to reduce osteolysis in inflammatory conditions and there may be scope for development of this therapy in the treatment of osteolytic metastases (94). It should be noted that RANKL-dependent and RANKL-independent mechanisms of osteoclast formation from mononuclear phagocyte populations are not mutually exclusive mechanisms and that there is likely to be considerable crossover under conditions of pathological bone resorption.

With regard to tumor osteolysis involving both these mechanisms, the vitronectin receptor αVβ3 an extracellular matrix receptor that recognises osteopontin, fibronectin and other matrix proteins via the Arg-Gly-Asp sequence is likely to be involved. Osteoclasts express αVβ3 and this receptor may also be a suitable target for pharmacological treatment with anti-αVβ3 agents. αVβ3 is expressed at high levels by metastatic cancer cells and endothelial cells, and promotes tumor cell survival, protease production and tumor angiogenesis (95).

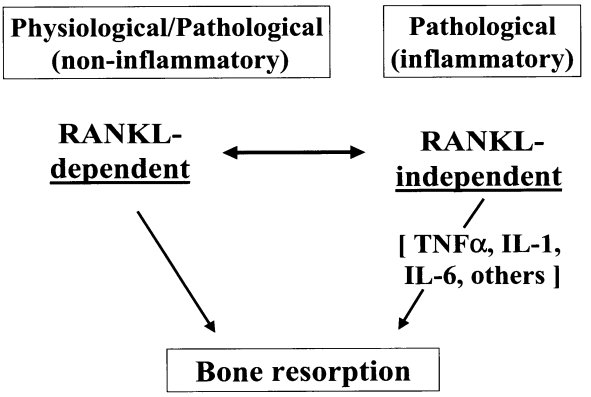

Figure 5. Scheme of RANKL-dependent and RANKL-independent (inflammatory cytokine-induced) osteoclast formation.

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Chapter 7

THE MALIGNANT HIERARCHY IN MULTIPLE MYELOMA: RELATIONSHIPS BETWEEN MALIGNANT CELLS AND BONE DISEASE

Linda M. Pilarski, Tony Reiman, Patrick M. Pilarski, F. William Orr and Andrew R. Belch
University of Alberta and Cross Cancer Institute, and University of Manitoba, Alberta, Canada

1. INTRODUCTION

Multiple myeloma (MM) is an incurable cancer of the bone marrow (BM), with survival of 3-4 years. MM accounts for 1% of all cancers, and 19% of deaths from hematological malignancies. It is characterized by monoclonal immunoglobulin (mIg) in the blood, lytic bone lesions, and monoclonal plasma cells in the BM. These plasma cells are responsible for most symptoms of MM, but considerable work confirms that the MM clone also includes earlier stage B lymphocytes. The MM clone is characterized by an immunoglobulin variable region gene rearrangement (IgH VDJ), termed "clonotypic". The IgH VDJ rearrangement that defines the MM clone in each patient is unique, thus providing an unequivocal molecular signature for MM cells regardless of, for example, genetic abnormalities, phenotype, morphology or physical location. Although the phenotypic properties of the MM clone are altered as the disease progresses and genetic heterogeneity increases, the clonotypic IgH VDJ signature remains constant, exhibiting rigid intraclonal homogeneity of sequence (1-3). With the exception of plasma cells, the distribution of the myeloma clone *in situ*, and its relationship to bone lesions within the BM is largely unknown. Because it is at best difficult, and at worst impossible to morphologically or

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phenotypically distinguish the earlier stage members of the MM clone from normal B lineage cells, detection of the IgH VDJ MM signature is essential as the distinguishing characteristic of individual MM cells participating in the apparently synergistic relationship between bone and malignancy.

The MM clone is highly heterogeneous, including multiple differentiation stages (Figure 1 A, B). The originating events and the subpopulations that promote bone destruction and osteoclast activity *in vivo* are unknown. Although provocative reports exist, there has been no characterization of the architecture of the MM clone *in situ* as it relates to physical relationships between subpopulations of MM cells and developing bone lesions. The widely applied reductionist approach involving analysis of cellular interactions *in vitro*, particularly those involving bone metabolism, must be extended to an *in vivo* context, to analyze the impact of therapy on these bone/malignancy inter-relationships. This is particularly true for agents such as bisphosphonates, which appear to have a profound impact on MM patients and possibly on the MM clone as well. Although nearly all MM patients are treated with bisphosphonates, there have as yet been no longitudinal studies in patients to define the characteristics of bone metabolism and tumor architecture during treatment with chemotherapy plus bisphosphonates *in vivo*. A careful analysis of bone disease in MM may lead to improved treatment options for MM patients by enabling the development of novel therapies to disrupt the synergistic cycle of tumor/bone interactions, halt bone damage and improve survival for patients with this deadly cancer.

Some MM patients are diagnosed at very early, largely asymptomatic stages of disease; these are termed indolent or smoldering MM and are usually left untreated until the disease progresses to more aggressive stages. Many patients with overt MM respond to melphalan/prednisone or vincristine/adriamycin/dexamethasone (VAD), but nearly all relapse and become refractory to treatment (4, 5). Autologous stem cell transplants achieve some complete remissions and prolong survival (6-11), but are not curative. Allogeneic transplantation has a high treatment-related mortality and few patients are eligible (10, 12). MM may arise through progressive acquisition of genetic abnormalities (13-15), and is characterized by complex chromosomal abnormalities (16). Although MM pathology is thought to result from plasma cells, no correlation is detectable between decrement in serum mIg or plasma cell kill after conventional chemotherapy and patient survival (17, 18). A modest increase in survival is found after cytoreduction and autologous transplantation (6). Generative potential within the MM clone may derive from less differentiated components.

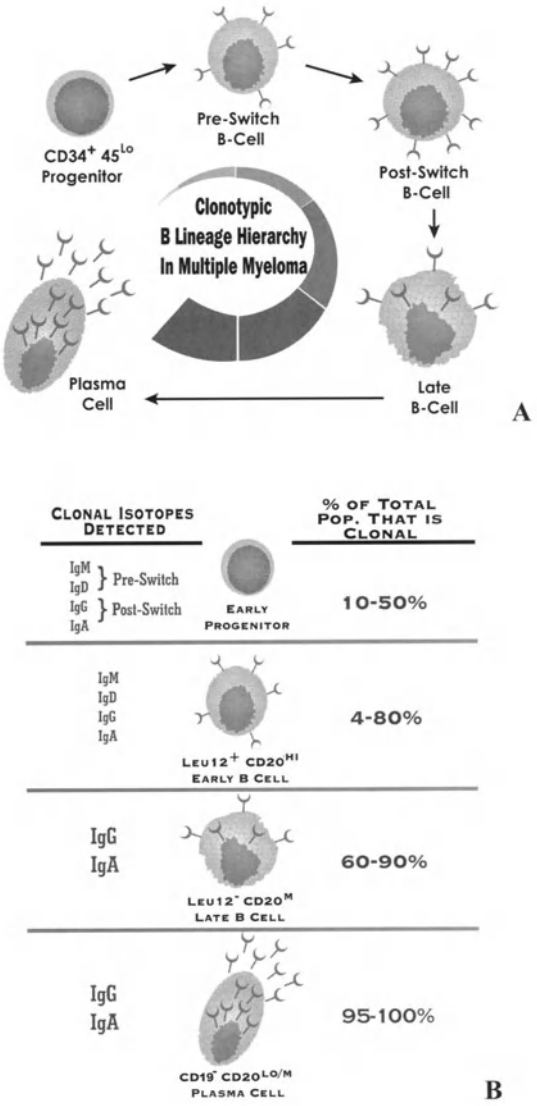

Figure 1. The MM hierarchy includes B lineage cells at all stages of B cell differentiation. For all stages shown in the diagram, clonotypic transcripts are detectable. However even for transcripts detected among very early stages including the MM CD34+ progenitor-like fractions and pre-switch MM B cells, the clonotypic IgH VDJ is maintained as a sequence that is identical to that expressed by end stage plasma cells. For all MM B cell stages detected, the VDJ is somatically mutated suggesting a post-germinal center, memory B cell phenotype. A) Developmental relationships within the MM hierarchy. B) Relative frequency on clonotypic cells within each phenotypically defined subset.

A variety of evidence suggests that MM represents a hierarchy of monoclonal B lineage cells in the blood and BM (1, 19-25) that includes late stage B cells and plasma cells (19, 21, 25), pre-B-like cells (26) and $sIgM^+$ pre-switch B cells (27-29) (Figure 1). B cells expressing the MM idiotype are detectable in blood (30-35), and circulating clonotypic MM B cells have been reported (25, 36-39). Our work shows that MM includes circulating B cells that persist despite chemotherapy and may mediate relapse (1, 19, 20, 22-25, 40, 41, 42-62). Even though MM plasma cells are often severely depleted in response to therapy, bone lesions usually persist, suggesting a failure of therapy to restore normal bone remodeling and the involvement of clinically cryptic components of the MM clone.

Several observations imply that early stage components of the MM clone are clinically relevant:

a) Xenografted early stage MM B cells give rise to lytic bone disease, clonotypic progeny and are self-renewing in murine BM (53).
b) Hematopoietic progenitor fractions of G-CSF mobilized blood, comparable to those used for autotransplants and shown to include clonotypic MM components, and MM B cells, engraft the myeloma clone and generate bone lesions in xenografted mice (63, 64).
c) Clinical purging of MM B cells from stem cell grafts used for autologous transplantation leads to prolonged survival (65).
d) Chemotherapy-resistant, IgM expressing components of the MM clone correlate with advanced disease at diagnosis and with significantly reduced survival (40).

We have characterized an abnormal population of drug-resistant, clonotypic B cells in the blood of MM Patients (1, 19, 21, 22, 40, 53, 56). Most circulating clonotypic MM B cells are surface Ig^+ (sIg) and express pan-B cell markers. By a variety of measures, including expression of IL-6 and IL-6R (66), they appear to be activated late stage B cells (20, 21, 45, 52). MM B cells express a large constellation of receptors involved in adhesion to endothelium or extracellular matrix and in adhesion/motility (22-24, 45, 46, 50, 67) suggesting that they home to, and colonize, the BM. For all patients tested (more than 100), MM B and plasma cells overexpress the RHAMM oncogene (24, 44, 67), which may play a significant role in the extensive genetic instability exhibited by MM B and plasma cells (52). Circulating MM B cells, but not BM plasma cells, also express CD34 epitopes and CD34 transcripts (22), leading to the speculation that CD34 may be involved in their migratory behavior. Morphologically and functionally, MM B cells are distinct from plasma cells (24, 25, 45). Although most MM B cells appear to be late stage, activated lymphoblasts, a smaller clonotypic subpopulation appears to be comprised of early stage resting lymphocytes expressing pre- and post-switch isotypes (Figure 1)

(40). The heterogeneity seen among *ex-vivo* clonotypic B cells suggests an *in vivo* hierarchy of sequentially related differentiation stages (Figure 1). The extent to which these early stage clonotypic cells inhabit the BM, participate in the generation of plasma cells and contribute to bone lesions remains to be addressed. It is important to emphasize that the early stage members of the MM clone are morphologically "cryptic" in that their physical appearance and phenotypic profiles are largely normal, preventing their identification using morphological criteria as "morphologically abnormal" cells. Only their expression of the clonotypic IgH VDJ signature confirms their relationship within the malignant clone.

2. BONE DISEASE IN MM

Although *ex-vivo* analysis of MM has provided valuable insights into the disease process, fundamentally, MM is a disease of the BM. MM pathology occurs in the BM. It is likely that generative phases of disease are also BM-localized, and that physical relationships between MM components and the cells responsible for bone metabolism are biologically important. Bone disease is a central pathological complication of MM. Most patients have characteristic lytic bone lesions in trabecular bone, osteoporosis is common, histomorphometry shows frequent trabecular damage, and fracturing of fragile bone is frequent. Osteoclasts are present in abnormal numbers lining regions of damaged bone, and the most commonly held view is that factors elaborated by MM plasma cells are responsible for activation of osteoclast differentiation and bone resorbing activity. Osteoblast function has been less extensively characterized. Osteoblast recruitment was actually increased in MM as compared to monoclonal gammopathy of undetermined significance (MGUS), but appeared to be significantly reduced in patients with overt MM as compared to those with early MM (68). However this was coupled with increased formation of new osteoclasts and increased bone resorption. As noted by Bataille and colleagues (68), factors from the MM clone may led to increased osteoblast function, which in turn may further stimulate the MM clone through production of growth factors such as IL-6, leading to increased MM-promoted osteoclastic activity. Thus MM appears to be characterized by interlinked and synergistic pathways of tumor growth and bone erosion. It is widely believed that all of these effects are mediated by the end stage malignant plasma cells that inhabit the bone marrow in MM. Here, we argue that earlier members of the MM hierarchy contribute to the maintenance of MM bone lesions when the cancer is in minimal stages, and that the MM clone may directly contribute to the bone erosion that characterizes the disease. This view of MM pathology is supported by

observations such as those of Rondelli and colleagues who described the appearance of new bone lesions in patients who have achieved complete remission after transplant (69). Osteoclasts are known to arise from monocytoid progenitors. We speculate that circulating monocytoid MM B cells, shown to be clonotypic using single cell RT-PCR analysis of IgH transcripts, may function as osteoclastic progenitors, thus contributing in a direct fashion to MM bone damage. This is supported by known links between B cells and macrophages (70). If this view is correct, it means that the MM hierarchy comprises two distinct but clonally related arms, one that gives rise to MM plasma cells and their associated pathological complications, and one that leads directly to osteoclast activity and bone erosion.

In MM, analysis of the BM has been limited by the inability to distinguish clonotypic from non-clonotypic populations of cells *in situ*. Although the patterns of plasma cell localization have been well characterized in MM at diagnosis, there has been little or no analysis of BM architecture, cell-cell interactions, or the precise distribution of the MM clone within the BM. For other cancers (e.g., breast and prostate) some evidence exists to suggest that tumor cells themselves have bone resorbing activity (71-73). As indicated above, based on a variety of suggestive observations, malignant components of the MM clone may also have direct bone resorbing activity, particularly during stages of minimal disease. The need to design more effective modes of therapy demands that MM/bone interactions be more extensively characterized *in situ*.

2.1 MM Bone Disease

In complete skeletons from two persons clinically diagnosed with MM while still alive, lytic bone lesions were seen as sharply defined spheroid areas, having smooth borders and internal surfaces with no evidence of new bone formation (74). MM lesions are distinctive as compared to those of metastatic carcinoma (74). Osteoclasts are multinuclear, macrophage-like hematopoietic cells that destroy bone. Overall, osteoclast numbers are increased only in bone resorption lacunae closely proximate to plasma cells (75), with a high correlation between numbers of plasma cells and osteoclasts (76). Of interest, patients with dense infiltrates of plasma cells often have few bone lesions, suggesting the importance of factors other than plasma cells (77, Belch, unpublished).

For many patients, plasma cell infiltration is accompanied by osteolytic lesions, causing fractures and bone pain. In normal BM, plasma cells are associated with vascular structures (sinusoids, venules and around capillaries). In early MM, plasma cells are loosely dispersed between fat and

hematopoietic cells. In later stages, plasma cells form sheets that are distant from vascular structures (78). Although chemotherapy reduces the plasma cell mass, it does not improve histological bone lesions in areas where plasma cells remain detectable (79), even though skeletal invasion by plasma cells is decreased. For a subset of MGUS, increased bone resorption in the vicinity of plasma cells suggested ongoing progression to MM (75). Although one study using bone histomorphometry has found few differences between untreated or chemotherapy-treated patients having heavy infiltration with plasma cells (79), there are no published histological analyses of MM bone lesions over time or as a function of therapy-induced reductions in plasma cell infiltration.

Interestingly, a high bone mineral density (BMD) has been found in the forearm (which lacks marrow), although the femoral neck and lumbar spine had low BMD (80), consistent with the view that marrow-localized cells influence the reduced BMD of MM patients. No correlation was found between BMD and presence or extent of lytic lesions (80). In contrast, serum paraprotein and % plasma cells were the most important predictors of femoral neck bone loss (81). Another study found that responders to therapy, and hence patients getting high-dose therapy, tend to do better in the context of changes in bone density than nonresponders/patients on conventional chemotherapy (82).

The inability to reverse bone damage and the persistence of bone lesions, even in stages of minimal disease, suggests that clinically cryptic components of the MM clone persist despite therapy and perpetuate dysregulated bone metabolism. Our observations that bone lesions are readily detectable in xenografted mice having few or no detectable plasma cells suggests that early stage MM cells themselves may have the ability to dysregulate bone metabolism (53, 63, 64). The evidence cited above supports the hypothesis that early stage B lineage MM cells interact with bone and contribute to bone lesions, as well as providing compelling justification for analysis of early stage members of the MM clone and their direct/indirect relationships to bone damage in MM.

2.2 Lymphoid Cells and Bone Lesions

Although lymphoid cells appear to influence bone pathology, there are no published studies to characterize the potential influence of lymphoid members of the MM clone on bone pathology. The fairly limited number of studies that have identified plasma cells in proximity to osteoclasts and bone lesions did not analyze lymphocytes that may have also been proximate, and in any case would have lacked the tools to determine whether or not any proximate lymphocytes were components of the MM clone. Reports of B

cell malignancies mimicking the bone disease found in MM indicate that B cells can promote lytic bone lesions (83). Provocatively, in BM with a nodular distribution of plasma cells, lymphocytes were found at the center of nodules, with mature plasma cells located in the marginal regions (78). In MM, frequent lymphoid *nodules* were associated with fewer lytic bone lesions and better prognosis (78, 84), but MM patients with diffuse lymphocytosis in BM had a shorter survival (78). Post transplant/donor lymphocyte infusion, an MM patient was found to develop new bone lesions despite the apparent absence of plasma cells or serum M protein (69). Anecdotal macroscopic observations indicate that bone lesions may fail to heal when disease enters remission.

Bone lesions were found in mice xengrafted with clonotypic lymphoid cells or with hematopoietic cells, having little, if any, plasma cell engraftment within the murine BM (63, 64) suggesting a role for lymphoid cells in the generation of bone lesions. Our hypothesis is that bone lesions persist despite successful chemotherapy because clonotypic MM B cells remain (23, 25, 41, 42) and continue to promote bone disease. The literature is conflicting in assessing healing of bone lesions in MM (77, 80-82, 85, 86). Some reports indicate that bones can and do heal in association with response to therapy, with an increase in bone mineral density as measured by X-ray, CT or MRI (77, 80, 82, 86), although bone lesions do not improve (85). However, the prognostic significance of this is unclear, with two groups showing that patients having normal X-rays or whose lesions heal on X-ray do worse (87, 88), perhaps because their disease is more diffuse, and two concluding that patients do better if the lesions heal (86, 89). Diamond et al. found that bone mineral density decreased over the course of conventional chemotherapy (81). Our local experience is that bone lesions can improve but that the improvement tends to be modest (TR, unpublished); usually there is no improvement seen when repeat x-rays are performed. Morphometric analysis of patients treated with chemotherapy suggest a lack of healing at the microscopic level (79). The relationship to the MM clone of lymphocytes that are proximal to regions of bone damage has not been examined, nor have the beneficial effects of bisphosphonates been correlated with a possible impact on lymphocytic members of the MM clone.

3. LYTIC BONE LESIONS IN MM PATIENTS

Although numerous studies have characterized the cells involved in bone metabolism *in vitro* or in animal models, very little, if any, work has analyzed the bone changes *in vivo* that accompany human MM as the disease waxes and wanes, ultimately progressing to extensive bone damage and

death. Bone damage is directly responsible for the adverse quality of life for MM patients and is a primary symptom of the disease.

Two potent intravenous bisphosphonates, pamidronate or zoledronic acid are most frequently used to reduce skeletal related adverse events in nearly all MM patients (90). Bisphosphonates adsorb to bone mineral and inhibit bone resorption by inhibiting osteoclast activity (91, 92). They appear to break the cycle of osteoclast bone destruction promoted by the MM clone, and significantly reduce bone fractures, but the mechanisms of action are far from clear. Evidence is accumulating to suggest that they act by inhibiting cytokines (93-95), and may alter micro-environmental interactions in MM (91). Finally, bisphosphonates may directly target the MM clone as well as inhibiting osteoclast activity (96). Patients not receiving bisphosphonates continue to lose bone during chemotherapy, probably in part due to the steroid therapy, whereas patients receiving bisphosphonates tend to gain in bone density (80, 97). However there was also a lack of correlation between bone density and the presence/extent of lytic lesions. Oral risendronate was shown to incease spinal bone mineral density, decrease serum markers of bone resorption, normalize bone turnover, and reduce histomorphometric measures of bone loss (77).

Some patients with extensive plasma cell infiltration lack detectable bone lesions, contrary to the expectation that factors from plasma cells stimulate osteoclast activity. Further, even though patients can enter "complete remissions" after autologous transplant, macroscopically detected bone lesions often do not heal, an unexpected observation if the dogma that plasma cells cause bone disease is correct, because transplant occurs at stages of minimal disease and plasma cell levels are severely depleted immediately post-transplant. These and other anecdotal observations suggest that bone pathology can be mediated through malignant MM components other than plasma cells. Analysis of human disease cannot adequately be described through analysis of model systems. A precise evaluation of the bone changes in MM and the cells responsible has to be founded on a thorough histomorphometric longitudinal analysis of bone pathology that records quantitative characteristics of bone damage at diagnosis, and as disease is treated, responds and relapses.

3.1 Histomorphometric Analysis of Bone in MM

Histomorphometry generates quantitative measures of bone volume, mineralization, and rates of remodeling that can be correlated directly with definition of molecular phenotypes of cells in the local bone microenvironment. Bone morphometry has been used extensively to define the pathophysiology of metabolic bone diseases, e.g., osteoporosis,

osteomalacia, renal osteodystrophy, and metastatic carcinoma (98). To date, histomorphometric analyses of bone biopsies from MM patients have not included molecular analysis of cellular components, and in particular, there are no studies to evaluate the presence of clonotypic B cells in MM BM.

Bone resorption dominates the biopsies of most myeloma patients (75, 99). In overt multiple myeloma, about 20% of biopsies demonstrate a generalized decrease in mean trabecular bone volume (osteoporosis; trabecular bone volume <11%), usually associated with increased osteoclastic resorption, reflected in an increased total trabecular resorption surface (75, 99, 100). In many biopsies, the mean trabecular bone volume is not reduced (or is even increased in about 5-10% of biopsies) as compared with controls and there may be localized lesions with a heterogeneous distribution of osteolytic and osteosclerotic areas (75, 79). It is important to note that histomorphometric abnormalities, e.g., increased osteoclastic resorption can occur in the absence of radiographic lesions, reflecting on the relative capabilities of radiographic and morphometric analysis to resolve the functional properties of bone.

Osteoclastic resorption surfaces, (total trabecular resorption surfaces and osteoclast number), are increased 2-3 fold in about 50% of untreated patients and about 35% of treated patients (79), especially in areas of bone adjacent to MM cells (75, 79, 101). Positive correlations have been reported between the levels of osteoclastic activity and the tumor burden (numbers of MM cells) as well as with the MM immunophenotype [IgA and pure Bence Jones MM appeared more osteoclastic than IgG cases ($P < 0.05$).] (75).

Increases in morphometric parameters that characterize bone resorption are generally correlated with an increase (0.75%) in the forming surface values (percentage of relative osteoid surfaces, relative osteoid volume, and the percentage of trabeculae that exhibit tetracycline labeling) (79). Part of the decrease in total bone volume may be due to decreased osteoblastic activity since, in some biopsies, osteoid seams have reduced thickness or volume (0.20%), and because there may be a low trabecular calcification rate (measured by tetracycline labeling) (75, 79).

Bone changes are absent or less severe in smoldering MM (102) and in MGUS (78, 103). In these biopsies, some studies have demonstrated foci of increased osteoclastic resorption, especially in areas populated by tumor cells (75, 78). In Bataille's study, MGUS patients with increased osteoclastic resorption surfaces had an increased relative risk of tumor progression, while MGUS patients with normal osteoclastic resorption parameters tended to remain stable (median follow-up = 28 months) (75, 104).

4. RELATIONSHIPS BETWEEN MM AND BONE DISEASE IN XENOGRAFT MODELS

4.1 MM Cells Express IL-6 and IL-6R

Interleukin-6 (IL-6) is a cytokine that has pleiotropic functions throughout the body. In MM, IL-6 has been implicated as an anti-apoptotic agent and also as a growth factor for the BM plasma cells (16, 95, 105-113). In addition, IL-6 activates the osteoclasts that mediate bone loss (93, 94, 114). IL-6 stimulates its own production from plasma and stromal cells in both autocrine and paracrine manners (105, 115). Circulating clonotypic MM B cells are able to produce and secrete IL-6 *ex vivo* (66), perhaps contributing to the cytokine network in MM. These cells strongly express CD31, the ligand for CD38, predicted to facilitate paracrine interactions with the ostoclast lineage and to exacerbate bone resorption in MM. Clonotypic MM B cells express surface IL-6 receptor α subunit, a prerequisite for autocrine and/or paracrine stimulatory loops. Our work suggests that clonotypic MM B cells have the potential for unregulated growth via autocrine stimulation and may contribute to the formation of bone lesions through paracrine stimulation of bone-resorbing cells.

4.2 Early Components of the MM Clone Impact Survival

MM is identified by clonotypic IgH-VDJ gene rearrangements and an M-protein isotype termed the "clinical" isotype. Transcripts encoding clonotypic pre- and post-switch IgH isotypes in MM were identified in peripheral blood (PBMC), BM and G-CSF mobilized blood stem cell collections (40). For 29 patients, 38 BM, 17 mobilized blood and 334 PBMC sequential samples were analyzed at diagnosis, pre- and post-transplant for 2-107 months. The clinical clonotypic isotype was readily detectable, and persisted throughout treatment. Overall, 82% of BM samples and 38% of PBMC samples also expressed non-clinical clonotypic isotypes. Clonotypic IgM was detectable in 68% of BM and 25% of PBMC. Non-clinical clonotypic isotypes were detected in 41% of mobilized bloods but clonotypic IgM was detected in only 12%. Patients with persistent clonotypic IgM expression had adverse prognostic features at diagnosis (lower hemoglobin, higher β2 microglobulin) and higher numbers of BM plasma cells compared to patients with infrequent or absent clonotypic IgM. Patients with persistent clonotypic IgM expression had significantly poorer survival than patients

with infrequent IgM expression ($p<0.0001$). In a multivariate analysis, persistent clonotypic IgM expression in the blood correlated independently with poor survival ($p=0.01$). In NOD SCID mice, xenografted MM cells expressed clinical and non-clinical post-switch clonotypic isotypes. Xenografted cells expressing clonotypic IgM were clonogenic and appeared to be self-renewing in murine BM (40). We speculate that myeloma may originate from a clonotypic IgM precursor cell that undergoes persistent isotype switching throughout the disease, leading to terminal plasma cell differentiation in the BM and perhaps initiating bone disease.

4.3 Circulating MM Cells Generate MM Bone Disease on Xenotransplant

The myelomagenic capacity of clonotypic MM cells during aggressive disease and in stages of minimal disease, defined by G-CSF mobilized autografts, were tested by xenotransplant (53). Intra-cardiac (IC) injection of immunodeficient NOD SCID mice with peripheral cells from 5 patients having aggressive myeloma led to lytic bone lesions, human Ig in the serum, human plasma cells and a high frequency of clonotypic cells in the murine BM. Intra-osseous (IO) injection of *ex vivo* MM cells directly into the murine sternal BM led to lytic bone lesions (Figure 2), BM plasma cells and a high frequency of clonotypic cells in the femoral BM. This indicates the spread of MM from the primary injection site to distant BM locations and subsequent initiation of macroscopically detectable bone damage.

Using a cellular limiting dilution PCR assay to quantify clonotypic B lineage cells, we confirmed that peripheral MM cells home to murine BM. The MM progenitor undergoes self-renewal in murine BM, as demonstrated by the transfer of human MM to a secondary recipient mouse. To obtain hematopoietic stem cells for autologous transplants, patients are treated with cyclophosphamide and G-CSF (a growth factor), a procedure termed mobilization. Mobilization causes stem cells to leave the BM and accumulate in the blood where they are harvested, cryopreserved and eventually reinfused after high dose chemotherapy. Mobilized blood stem cell autografts from 6/7 MM patients, taken at the time of mobilization or after cryopreservation, included MM progenitors as identified by engraftment of clonotypic cells and/or lytic bone disease in mice. This indicates that MM progenitors are mobilized into the blood, and are present even during stages of minimal disease. Such residual cells are likely to contribute to post-transplant relapse and to mediate persistent bone disease. The ability of mobilized autografts to generate MM after xenotransplantation implies that such progenitors are also myelomagenic when reinfused to patients, and suggests the need for an effective purging strategy prior to

transplant. We speculate that *in vivo*, these same MM cells home to, and colonize patient BM, thus perpetuating the cycle of homing to trabecular bone and bone damage that characterizes MM *in vivo*.

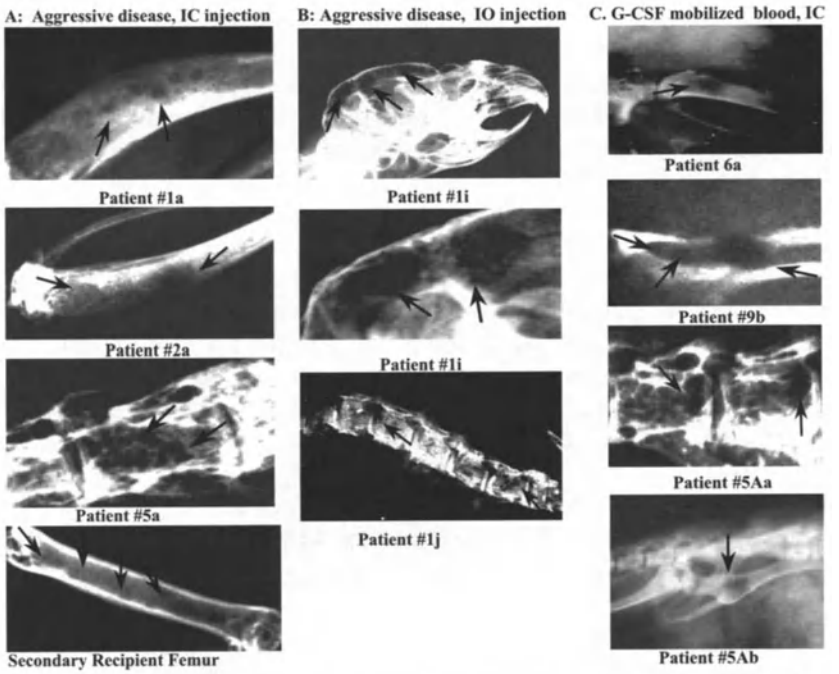

Figure 2. Lytic bone lesions in xenografted mice. IC - intracArdiac injection, IO - intraosseus injection. Patients listed as having aggressive disease had circulating plasma cells. Patients from whom G-CSF mobilized blood autografts were obtained were in minimal disease. Pilarski et al. Myeloma progenitors in the blood of patients with aggressive or minimal disease: Engraftment and self-renewal of primary human myeloma in the bone marrow of NOD SCID mice. Blood 95:1056-1065, 2000. Copyright American Society of Hematology, used with permission.

4.4 MM B Lymphocytes Generate Lytic Bone Disease in Xenografted Mice

Although MM plasma cells express CD138 (syndecan-1), clonotypic myeloma B cells are $CD19^{+}138^{-}$ in all patients tested. Using single cell RT-PCR and PCR, we showed that aggressive circulating lymphoblasts from an MM patient who developed Burkitt's-like lymphoblastoid leukemia were all clonotypic with an IgH VDJ sequence *identical* to that of autologous plasma

cells at diagnosis (64). Flow cytometric analysis showed leukemic B cells were $CD19^+$ 138^-. For both the myeloma and the leukemic phases of the disease, the clinical clonotypic isotype was IgA. Analysis of IgH transcripts indicates MM clonal dominance over normal components of the immune system at diagnosis and prior to terminal Burkitt's lymphoma-like leukemic disease. $CD138^-$ leukemic B cells were xenografted to irradiated NOD SCID mice via intra-cardiac injection. At 3-4 months post-engraftment, lytic bone lesions and clonotypic cells were detected in the BM of engrafted mice (64). Although a subset of human cells in the murine BM expressed CD138, a marker largely absent from *ex vivo* leukemic cells, the expression of CD45, CD19 and CD20 confirmed that engrafting cells were late stage B-cells rather than plasma cells. Thus, leukemic B-cells colonize the murine BM in a xenograft model, and disrupt normal bone metabolism leading to lytic bone lesions. This supports the hypothesis that clonotypic MM B-cells are reservoirs of disease that persist throughout therapy and play a significant role in dysregulated bone metabolism and bone damage. It also provides strong justification for the hypothesis that MM B cells are direct and/or indirect contributors to the cycle of bone homing and bone destruction in MM.

4.5 Xenografted Progenitor-like MM Cells Give Rise to Clonotypic MM Cells and Lytic Bone Lesions

The identity of MM precursor(s) is unknown, and the contributions to MM pathology of early stage components of the MM clone remain unclear. To address this question, the myelomagenic capabilities and contributions to lytic bone disease of CD34-enriched autografts were determined (63). Hematopoietic progenitor fractions from fresh or cryopreserved G-CSF mobilized blood from MM patients were obtained by sorting or enrichment, followed by RT-PCR analysis of clonotypic transcripts and/or their ability to transfer myeloma to immunodeficient mice. Highly purified $CD34^+$ progenitors from G-CSF mobilized blood of myeloma patients include on average, 24% clonotypic MM cells. $CD34^+$ progenitors also include 31% DNA aneuploid cells.

For 6/6 MM patients, enriched progenitors were myelomagenic on xenograft analysis, as measured by engraftment of clonotypic cells and/or the development of lytic bone lesions. The majority of mice (73%, range 60-100%) engrafted with enriched progenitors had lytic lesions in the skull, spine, pelvis and/or tibia. Bone lesions were found at distant locations in mice injected intra-sternally, further confirming the spread of disease that had originated from the enriched progenitors. No lesions were detected in irradiated control mice that had not received enriched progenitors. The

presence of bone lesions at distant skeletal locations confirms dissemination of myelomagenic cells throughout the skeleton, the preferential homing to bone sites and the generation of bone lesions either directly or indirectly from early stage, resting lymphocytes with clonotypic IgH VDJ.

The involved cells are likely to be post germinal center, memory B cells, given that clonotypic transcripts detected in sorted or enriched progenitor populations appear to be predominantly post-switch IgG or IgA cells with a somatically mutated IgH VDJ clonotypic sequence identical to that of autologous plasma cells. Unlike mice xenografted with leukemic plasma cells (53, 64), the lesions in mice injected with enriched progenitor fractions were less pronounced and clonotypic transcripts were less frequent (63). This may reflect different degrees of differentiation for MM precursors among leukemic plasma cells and those among earlier stage B cells, since end stage plasma cells have considerably higher levels of Ig and Ig transcripts which are more readily detected in our assays. Within the 1-3 month window evaluated here, MM precursors present among CD34- enriched progenitors may not complete differentiation to plasma cell stages, resulting in engrafted MM cells with lower transcript copy numbers.

These observations implicate early stage MM components in the generation of lytic bone disease. For many mice, lytic bone lesions were readily detectable even though clonotypic transcripts were below our levels of detection. This suggests that bone lesions and/or altered bone metabolism was induced in the absence of differentiation by MM progenitors to end stage plasma cells. By extrapolation, this work is supportive of a role for malignant lymphocytes in the generation of bone disease in MM, providing further justification for the hypothesis that MM B lymphocytes home to bone, disrupt normal bone metabolism and are significant participants in MM bone pathology.

Both plasma cells and lymphocytes have been reported to have prognostic significance in BM biopsies at diagnosis (78, 84). Lymphoid nodules are detectable in MM BM (78, 84), lymphocytic infiltrates persist after high dose chemotherapy (116), and plasma cell nodules have centrally located lymphocytes (78). Together with the evidence cited above, it seems possible that lymphocytes contribute to early stages of bone disease, as identified histologically, while plasma cells may be required to stimulate massive bone destruction that leads to lytic bone lesions identified macroscopically. It is unknown whether the lack of new bone synthesis in MM represents a systemic defect in osteoblast function, or if it represents a localized uncoupling of bone loss and gain caused by proximity to malignant cells.

5. FUNCTIONAL PROPERTIES OF LYMPHOCYTIC CELLS IN BM LESIONS

Although the evidence from our work and that of others clearly indicates that the MM clone includes a hierarchy of B lineage cells, the extent to which any or all of these cell types serve as generative reservoirs of human disease *in situ* remains ambiguous. The early members of the MM clone appear to be drug resistant components that persist throughout conventional and high dose chemotherapy. Although novel biologically based therapies such as thalidomide are proving very effective in MM, patients still relapse, indicative of drug resistant components despite achieving complete remission (as defined by undetectable levels of mIg). Drug-resistant B cell members of the MM hierarchy may be significant components of the process that ultimately leads to relapse. However as indicated above, it appears that multiple components of the MM clone have the ability to transfer disease and/or correlate with reduced survival. Taken together, work by ourselves and others indicates that progenitor function in MM occurs at several levels, and must be evaluated in terms of an ability to generate as progeny the plasma cells that contribute to bone pathology, and ultimately to patient death.

5.1 Plasma Cells

It is widely believed that the primary generative components of MM are the BM-localized plasma cells that cause symptoms and pathology, including bone disease, of MM. No matter which B cell maturation stages harbor progenitor function and which lead to osteoclastic bone resorption, the ultimate mediators of MM are likely to be BM plasma cells. For example, early stage MM cells may mediate osteoclast generation during stages of minimal disease while in aggressive disease the extensive plasma cell infiltration may stimulate clonally normal osteoclastic activity. Hypothetically, since plasma cells have such extensive ability to mediate disease pathology, if they are able to undergo even a minimal number of generative cycles, they will be detected as "myelomagenic" in model systems and in patients. We predict that plasma cells with progenitor function will rapidly generate detectable disease because they have already terminally differentiated to the pathological stage (as diagrammed in Figure 3). This prediction appears to be supported in both a Hu-SCID model in which BM plasma cells are injected directly into a human fetal bone implant under the kidney capsule of an immmunodeficient mouse (117, 118), and in our own work injecting leukemic plasma cells to NOD SCID mice (53). In both cases, the abundant plasma cells appear to rapidly engraft and cause

bone lesions. However, this cannot be a complete picture since in patients, the number of plasma cells in BM autografts does not appear to correlate with survival (4). In all of these examples where plasma cells have apparent progenitor function, it seems likely that aggressive plasma cell progeny cause death before any impact of therapy targeting/depleting earlier stage progenitors could be detected.

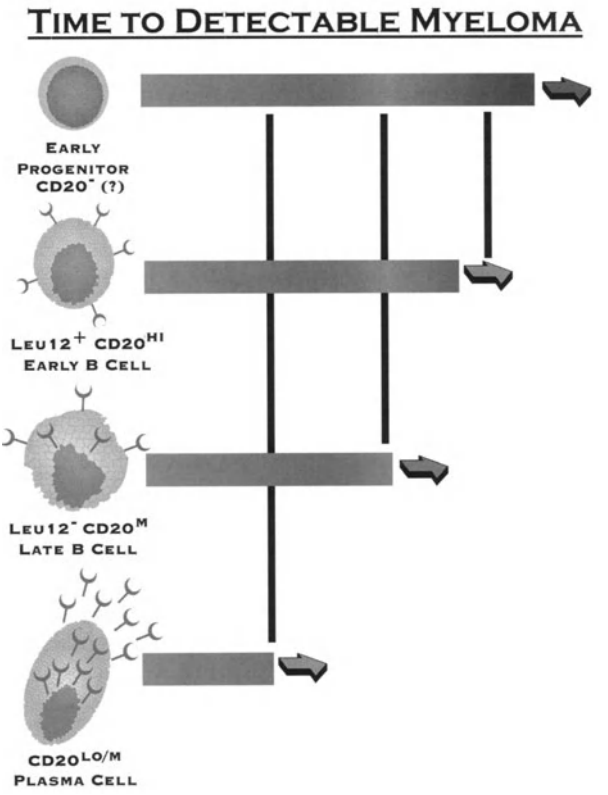

Figure 3. Heterogeneity in MM progenitor cells. The time to detectable disease depends upon the maturation stage of the progenitor fraction of MM cells. Early stage progenitors are predicted to exhibit a prolonged delay before disease becomes detectable, and to have extensive self renewal capabilities. Late stage progenitors are predicted to lead to rapid symptomatic disease, and to have quite limited self renewal capability. Since the plasma cell pool is predicted to be readily replenished by early stage progenitors, depletion of late stage progenitors is likely to be reversed by drug resistant earlier stage progenitors. Thus therapeutic attack must be targeted to both early and late stage disease components.

5.2 Mature B Cells

G-CSF mobilized blood from patients in minimal disease xenografted human MM to NOD SCID mice (53). For 9/9 mobilized blood preparations, <0.01% plasma cells were detected, but $CD19^+$ B cells were readily detectable by their clonotypic transcripts (63). For 6/7 patients tested, mobilized blood engrafted murine BM, as detected by clonotypic transcripts and bone lesions (53). Interestingly, mice xenografted with mobilized blood autografts had readily detectable bone lesions on X-ray, despite the absence of detectable plasma cells in their marrow. The frequency of mice with detectable clonotypic transcripts was also lower suggesting that differentiation had not yet reached abundant plasma cell stages, at which time transcript copy numbers would be extremely high and easily detectable. This work suggests the presence of a mature B cell progenitor stage, with the ability to cause disease, but by virtue of their mature B cell stage, a delayed ability to generate end stage plasma cells relative to progenitor-competent plasma cells (Figure 3). This is borne out by the analysis of leukemic B cells that developed in a MM patient (64). These circulating, clonotypic B cells were able to engraft BM and generate lytic bone lesions in the absence of visible symptoms or detectable plasma cell progeny. For the normal immune system, the concept that earlier progenitors require a longer time to generate end stage effector cells is widely held and supported by immunological experiments (119-121).

Pre-switch MM cells also engraft immunodeficient mice (40), leading to pre-switch clonotypic cells in murine BM, but the extent to which these cells contributed to bone lesions in such mice could not be evaluated. However, for xenografted circulating cells from one patient, the *only* clonotypic transcripts detected in BM and spleen were of the IgM isotype (Figure 4). All three xenografted mice had detectable osteolytic lesions, suggesting these originated from engraftment of pre-switch MM B cells derived from the peripheral blood xenograft. In contrast, all clonotypic isotypes were detected within vertebral masses that arose in these mice (Figure 4).

A large concentration of human MM cells may be a prerequisite for the expression of post-switch isotypes (as detected in the vertebral masses). The absence of post-switch clonotypic isotypes in lymphoid tissues may reflect a lesser degree of clonal expansion by pre-switch MM precursors, as compared to the macroscopically greater expansion in the vertebral tumor masses, consistent with observations of Hodgkin and co-workers showing that isotype switching increases with the number of clonal divisions (122). As well, a high concentration of MM cells may be needed to maintain and/or to generate cells of post-switch clonotypic isotypes, perhaps reflecting production of human cytokines and/or a requirement for cellular interactions.

Direct evidence that pre-switch progenitors may have a strong influence on MM progression comes from our longitudinal analysis (40) which showed a strong correlation between persistent, and thus drug-resistant, pre-switch MM cells and reduced survival ($p<0.0001$). Thus in patients, where the pre-switch MM cells have a prolonged time in which to undergo clonal expansion and differentiation to plasma cell stages, a clinical impact is detected. In this context, the relationship between pre-switch MM cells and bone disease is unknown. However the data of Figure 3 suggests they may cause bone lesions. This reinforces the idea that evaluation of progenitor function in MM must take into account the parameters of time and maturation stage.

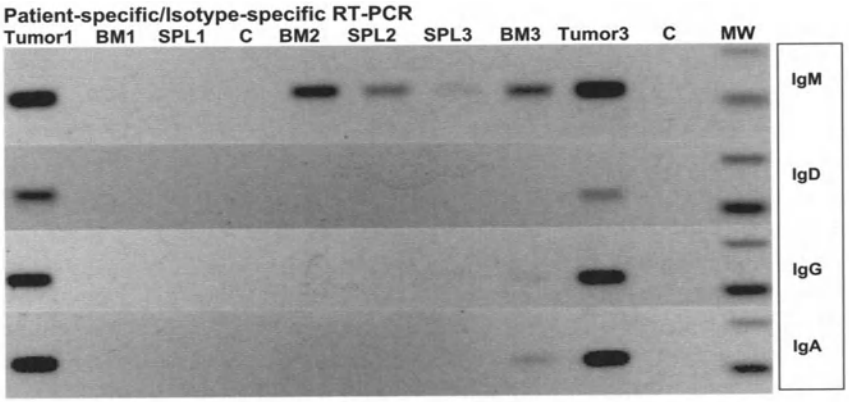

Figure 4. Clonotypic IgM transcripts in spleen and BM of mice xenografted with PBMC from a relapsed MM patient. Three xenografted mice developed visible symptoms and were sacrificed at about 3 months post-engraftment. Large vertebral masses were found in all three mice. T- tumor vertebral mass, BM- bone marrow, Spl- spleen, C-PCR control lanes. Nested PCR used VHLeader/constant region primers followed by CDR2/CDR3 patient-specific primers (22).

5.3 CD19$^-$ MM Progenitors

We have shown by a variety of measures that CD34$^+$ hematopoietic progenitor populations include clonotypic MM cells, but lack detectable plasma cells (63). These CD34-enriched fractions composed of normal progenitors and MM cells engraft MM clonotypic transcripts to NOD SCID BM and lead to lytic bone lesions. Since CD19 mAb is included in the cocktail of antibodies used to deplete lineage committed cells, the MM

progenitors being detected here appear to lack CD19 but are clearly mature memory B cells by virtue of a somatically mutated IgH VDJ in the inoculated cells and in their progeny in the engrafted mice. These engrafted mice did not exhibit visible disease symptoms and clonotypic transcripts were detectable in only 18% of mice suggesting that their progeny were not plasma cells but rather earlier stage cells with low transcript copy numbers. The dilemma in MM, illustrated in Figure 3, is that if only plasma cells are eradicated, the MM B cell progenitors described above will still mediate bone disease and will eventually restore the plasma cell component leading to re-emergence of disease, continuation of bone erosion and relapse. If only the early stage progenitors are eradicated, the plasma cells remaining in the patient will lead to relapse and death. This means that for MM, combination therapeutic strategies designed to target multiple components of the MM clone will be required to halt the progression of the MM clone and improve patient survival.

5.4 Myeloma and Bone Metabolism

A variety of cytokines control bone metabolism, with osteoblasts creating new bone and osteoclasts destroying bone (114, 123). The final mediators are osteoprotegerin-ligand [OPGL, a tumor necrosis factor (TNF)-related cytokine], RANK, the cell surface receptor for OPGL, and OPG a secreted TNF receptor family member (124). OPG sequesters OPGL, thus preventing its interaction with RANK, to down-regulate osteoclast differentiation (125). Thus, osteoclastogenesis is inhibited by OPG and promoted by OPGL (124, 126). OPG functions as an inhibitor of osteolytic bone disease in an animal model of MM (127). OPGL (RANKL) is detectable as a surface membrane receptor on normal B cells (128) and MM plasma cells (129). In this context it is interesting that MM B cells bind OPG -FITC (a recombinant OPG lacking the heparin binding domain) (AJ Szczepek and LM Pilarski, unpublished), suggesting that they may be stimulated in some way through binding of OPG and may play a significant role in disordered bone metabolism. Other key cytokines involved in osteoclastogenesis are IL-6 and colony-stimulating factor-1 (CSF-1) (114). Plasma cells may exacerbate this process by syndecan-1 mediated sequestering of OPG (92), and/or production of OPGL, thus increasing the active concentrations of OPGL. Although controversial, several groups have detected RANKL on myeloma plasma cells (129, 130). Serum OPG is reduced in MM patients having lytic bone disease (131-133), again suggesting an intimate relationship between the MM clone and bone disease. MM B lymphocytes constitutively express IL-6 and IL-6R (66), as well as expressing CSF-1 and CSF-1R (LMP, unpublished), suggesting they may play an important role in

osteoclastogenesis in the BM. Possibilities are a) they themselves are osteoclast progenitors, as predicted by reference (134), under autocrine growth promotion, or b) they produce paracrine factors that stimulate osteoclast differentiation.

It has been speculated that members of the MM clone may differentiate to osteoclasts (134). Our own work raises the possibility that MM clonotypic B cells, which have monocytoid properties and morphology, may differentiate to osteoclasts or perhaps themselves directly participate in bone resorption. This notion is supported by work showing that MM PBMC include unidentified osteoclast precursors that give rise *in vitro* to osteoclasts with bone resorbing capability (135). Provocatively, CD56 (NCAM) was strongly expressed by ostoclasts and osteoblasts, but only in MM with lytic bone lesions did $CD138^{+}$ plasma cells coexpress $CD56^{hi}$ (lymphocytes were not evaluated in this study) (136), suggesting that homophilic binding between these cells, mediated by CD56, was important for both decreased bone formation and increased bone resorption. In this context, it may be significant that high levels of CD56 are also expressed by MM B cells (25), which have the potential for autocrine secretion of osteoclastogenic factors that may also inhibit osteoblast function (66). Finally, recent work shows that early stage normal B cells have the potential to differentiate into osteoclasts (128), lending support to the view that malignant B cells may retain this capability and raising the possibility that the clonotypic IgM-expressing MM B cells described above (40) may influence survival via an impact on bone metabolism. The view that MM B lymphocytes may be directly responsible for bone destruction is supported by our xenograft analysis of human MM (53, 64). Lytic bone lesions were detected in mice xenografted with early stage human cells that had little, if any, plasma cell infiltration in the murine BM (53, 63, 64), suggestive of a role for lymphocytic members of the MM clone in this process.

6. CONCLUSIONS

Although the idea that MM cells themselves may directly contribute to bone damage in MM, as opposed to indirectly through stimulation of normal osteoclastogenesis, challenges current dogma regarding the pathology of MM, it is important to test this hypothesis. A central issue is to determine the extent to which, if any, MM cells themselves contribute directly to bone damage since this is such a significant aspect of MM pathology. The pathological effects of monocytoid, clonotypic B cells in MM are unclear, but they have many of the properties thought to characterize osteoclast progenitors, including a cytokine profile consistent with autocrine

stimulation and osteoclastogenesis. It will be important to clarify the functional properties of this arm of the malignant clone, particularly as it relates to bone resorption. This type of analysis is likely to lead to new perspectives on myeloma pathogenesis. If so, the implications of this new perspective on disease biology for the design of novel treatments to better attack MM and its consequent bone disease are profound.

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Chapter 8

ANGIOGENESIS AND LYMPHANGIOGENESIS IN METASTATIC BONE DISEASE: A MATTER OF NETWORKING

Gabri Van Der Pluijm and Jeroen Buijs
Leiden University Medical Cente,rDepartment of Endocrinology C4-R86, Leiden, The Netherlands
Leiden University Medical Center,Department of Endocrinology C4-R, Leiden, The Netherlands

1. INTRODUCTION

In healthy adults, (lymph)angiogenesis is limited to processes of repair (wound healing) and is restricted to the female reproductive tract (ovulatory cycle). In normal tissues, vascular quiescence is maintained by the dominant influence of endogenous angiogenesis inhibitors over angiogenic stimuli. In pathologic conditions, however, this delicate balance can be disrupted and lead to initiation and stimulation of blood or lymphatic vessel formation ('angiogenic switch'). Neovascularization or angiogenesis -the formation of new blood vessels from pre-existing ones- is vital for the growth of tumors, providing a lifeline for sustenance and waste disposal. It is, therefore, tempting to speculate that interactions between osteotropic cancer cells and the (a)cellular bone marrow stoma lead to differential expression of (lymph)angiogenic factors and their inhibitors in the bone microenvironment, thus explaining -at least in part- the observed bone tropism by a limited number of carcinomas.

Osteotropic cancers can generate a microcirculation comparable to processes involved to the development of a vascular system during embryogenesis. In this chapter, we will discuss the role of angiogenesis, lymphangiogenesis, postnatal vasculogenesis and vasculogenic mimicry in

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growth, invasion and metastasis of osteotropic cancers, particularly those of the breast and prostate.

2. THE VASCULAR SYSTEM DURING EMBRYONIC DEVELOPMENT AND POST-NATAL LIFE

The processes involved in the formation of the vascular system during embryonic development can serve as templates for better understanding the basic principles in the acquisition of a microcirculation in tumors. The first organ system to develop in the embryo is the vascular system. Development of the blood vessels starts with the formation of a primitive vascular plexus by endothelial cells that are generated from mesenchymal precursors (1-7), a process that is called *vasculogenesis* (Figure 1). The hallmark of vasculogenesis is the differentiation of a pluripotent stem cell into a hemangioblast in the blood islands of the yolk sac. The hemangioblast gives rise to various blood cells, and cells from the endothelial lineage.

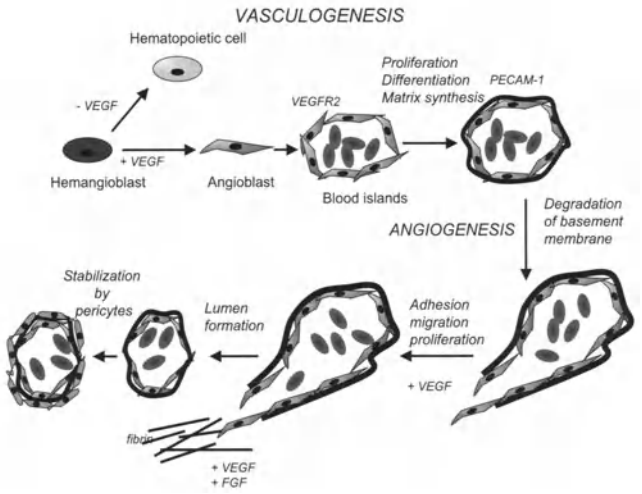

Figure 1. Vasculogenesis and angiogenesis. Blood and lymphatic vessels are initially formed through differentiation and proliferation of precursor cells (vasculogenesis), which occurs in the yolk sac. After establishment of the primary plexus, this plexus is remodelled through a process, which is called angiogenesis and involves degradation of the basal membrane to enable migration, proliferation and tube formation. (Figure adapted from M.M.L. Deckers, Building Bones. The role of VEGF-A in embryonic bone formation. PhD Thesis, 2001).

The presence or absence of vascular endothelial growth factor A (VEGF-A) determines the cell fate of the precursors. In the absence of VEGF-A, the inner cells of the blood islands become hematopoietic precursors (VEGF Receptor 2 (VEGFR2 negative cells) that will develop further into mature hematopoietic cells in the blood islands (primitive hematopoiesis). Later in development, hematopoiesis shifts to the fetal liver and eventually to the bone marrow. In the presence of VEGF-A, however, the outer cells of the blood islands differentiate into angioblasts (VEGFR2 positive cells) which mature into endothelial cells (7). Blood vessel development depends on members of the VEGF family of proteins (Figure 2). This family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PlGF), which bind and activates cell surface receptors tyrosine kinases. The VEGF receptor (VEGFR) family includes VEGFR1 (also known as Flt-1), VEGFR2 (KDR, Flk-1) and VEGFR3 (Flt-4). Neuropilins 1 and 2 (NP1 and 2) are another class of high-affinity non-tyrosine kinase receptors for VEGFs on endothelial and neuronal cells (3, 5, 8-13). Other factors that are involved in blood and lymphatic vessels are the angiopoietins and Tie-receptors, ephrins, and platelet-derived growth factors or PDGF (14-16). It is important to note that the presence of the angioblast is not restricted to embryonic development, since it remains circulating in the peripheral blood during adulthood.

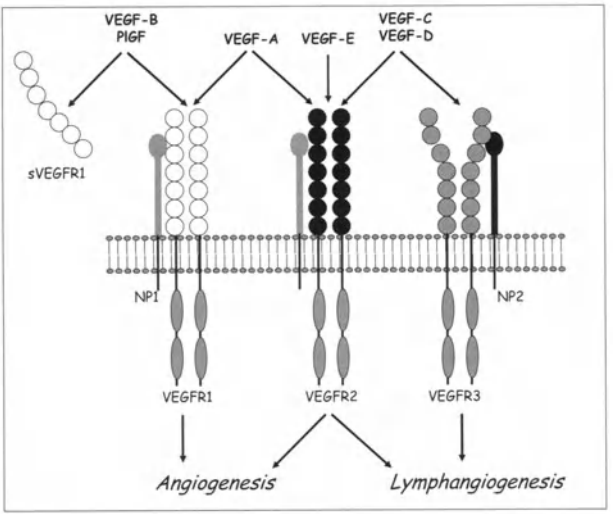

Figure 2. The vascular endothelial growth factors (VEGFs) and their receptors. PlGF=Placenta growth factor; NP=neuropilin; sVEGFR1=soluble VEGF receptor-1.

Angiogenesis or neovascularization, the formation of blood capillaries from pre-existing blood vessels, extends and remodels the primitive vasculature giving rise to a complex vascular network (Figure 1). Angiogenesis is a multi-step process that is regulated by soluble factors as well as by cell-matrix interactions (8, 12, 13, 17-20). Activation of endothelial cells by angiogenic stimuli derived from non-endothelial cells results in expression of proteolytic enzymes and site-directed proteolytic activity. After degradation of the basement membrane, endothelial cells adhere to the surrounding matrix, migrate towards angiogenic stimuli and invade the surrounding tissue. Proliferation of endothelial cells in the sprouting capillary results in extension and elongation of the sprout and eventually a lumen is formed. Several sprouts will join to form a loop in which blood can circulate, resulting in vascularization of the tissue. Finally, pericytes are recruited to stabilize the newly formed vessels. Angiopoietins 1 and 2, PDGF, and members of the transforming growth factor-β (TGF-β) superfamily are crucial to this final step of angiogenesis. Angiopoietin 1 and PDGF are involved in the reciprocal communication between endothelial cells and pericytes. In contrast, angiopoietin 2 acts both as an inhibitor and stimulator of angiogenesis, while, in the presence of VEGF-A, angiopoietin 2 stimulates detachment of peri-endothelial cells, thereby promoting angiogenesis.

In addition to blood vessels, lymphatic vessels belong to the vascular circulatory system. Although the lymphatic vessels were discovered in the early 17^{th} century, relatively little is known about their formation, maintenance, and their exact role in disease (8, 12, 18, 20, 21). This is mainly due to the lack of specific markers. Only recently have researchers identified molecules whose expression is restricted to the lymphatic endothelium. It has been proposed that isolated lymph sacs originate by endothelial budding from embryonic veins (8, 12, 18, 20, 21 22). The peripheral lymphatic system spreads from primary lymph sacs by endothelial sprouting into the surrounding tissues or organs. Alternatively, it has also been suggested that lymph sacs arise from the mesenchyme from precursor cells (lymphangioblasts) independent of the veins but that they form venous connections later. Although lymphatic vessels share several properties with blood vessels (e.g. lining by endothelium), there are clear differences. Blood vessels have a continuous or fenestrated basement membrane and tight interendothelial junctions, which make the vessel wall selectively permeable to cells, fluids, and molecules, whereas lymphatic vessels constitute a relative barrier for interstitial fluid (8, 12, 18, 20, 21). In addition, lymphatic vessels have complex overlapping intercellular junctions and specialized anchoring filaments which hold the vessel open as tissue pressure rises. Liquids, macromolecules, and migrating cells pass through the blood

capillary endothelia, enter the tissues, and are gradually absorbed into the lymphatic system. The fluid is transported via the lymphatic capillaries into the collecting vessels and through the lymph nodes eventually returning to the circulation (8). The blood vascular endothelium is a relatively leak-proof, nonthrombogenic surface, with tightly regulated flow and intraluminal pressure, whereas the lymphatics, in contrast, are a low-flow, low-pressure system in close contact with the extracellular matrix (8, 12, 18, 20, 21).

3. ANGIOGENESIS IN TUMOR GROWTH AND METASTASIS.

Tumor blood and lymphatic vessels can grow by sprouting, intussusception or by incorporating bone marrow-derived endothelial precursor cells into growing vessels (postnatal vasculogenesis, *de novo* formation of blood vessels by hemangioblasts) (8, 18, 20, 22). Angiogenesis is essential for tumor growth and metastasis. Angiogenesis is a complex, multistep process that involves dissolution of the basement membrane of the vessel and extracellular matrix degradation, migration and proliferation of endothelial cells, capillary differentiation, stabilization and anastomosis.

Inducers and inhibitors of endothelial proliferation, migration and differentiation tightly regulate these processes. In the adult, the vascular network is relatively stable and vascular turnover is extremely low and is considered to be nearly quiescent. With the exception of the female reproductive cycle, angiogenesis in adults occurs only under pathological conditions such as wound healing and tumor growth, when the balance is shifted in favor of angiogenesis either by increased levels of angiogenic inducers or decreased levels of angiogenic inhibitors. In malignancy, tumor cells can switch from an angiogenesis-inhibiting phenotype to an angiogenesis-stimulating phenotype, a process which is referred to as the *angiogenic switch* (23-25).

Angiogenesis is essential for tumor growth as it provides oxygenation and nourishment of the tumor and allows removal of waste products. Angiogenesis thus allows the tumor cells to express their critical growth advantage and also permits the establishment of continuity with the existing vasculature of the host. In addition, increased angiogenesis coincides with increased tumor cell entry into the circulation and thereby facilitates metastasis. A strong correlation has been found between the density of microvessels in primary breast and prostate tumors and nodal metastases and patient survival, as in other malignancies (17, 26-49).

Tumors produce and secrete a variety of pro-angiogenic molecules, incuding members of the VEGF family (Figure 2). VEGF-A is a specific

endothelial cell mitogen and inducer of angiogenesis. It also potently induces vascular permeability to macromolecules and is, therefore, also known as vascular permeability factor (VPF). VEGF-B binds to VEGFR-1 whereas VEGF-C binds to VEGFR-3 (flt-4).The latter receptor is specific for lymphatic endothelium and VEGF-C stimulates the growth of lymphatic vessels. VEGF-D is closely related to VEGF-C and bind to the same receptor. Proteolytically processed VEGF-C and D are also able to bind to VEGFR-2 but have lower affinity and are, therefore, weakly angiogenic (6) (Figure 2).

In addition, it is becoming increasingly clear that various malignant tumors can acquire a microcirculation by recruiting circulating endothelial progenitor cells or hemangioblasts from peripheral blood (Figure 1). Xenotransplant models have convincingly demonstrated that postnatal vascularization in various cancers does not rely exclusively on sprouting from pre-existing blood or lymphatic vessels. Instead, endothelial progenitors circulate from the bone marrow to incorporate into and thus contribute to postnatal physiological and pathological neovascularization, which is consistent with postnatal vasculogenesis (2-4, 6, 9, 50-52). Tumors are, therefore, capable of stimulating *de novo* formation of blood capillaries that can subsequently fuse with the existing vasculature, thus connecting the tumor to the vasculature of the body.

4. THE EMERGING ROLE OF LYMPHANGIOGENESIS AND TUMOR METASTASIS

Metastasis of osteotropic cancers, like breast and prostate carcinoma, occurs in part through the lymphatic system, and the extent of lymph node involvement is a key prognostic factor for the disease. While the importance and prognostic significance of the ability of tumor cells to stimulate angiogenesis has been firmly established, the significance of lymphangiogenesis has not been well documented. This has been mainly due to the lack of consistent data with reliable lymphatic endothelial cell markers. However, the recent discovery of novel lymphatic endothelial cell markers (e.g. prox-1, LYVE-1, VEGFR3 and podoplanin) have led to the demonstration of newly formed lymphatic vessels within the bulk of the tumor in animal models and human neoplasms, and the characterization of the VEGF-C/VEGFR-3 pathway (20, 53-57).

In vivo studies have shown that the soluble protein growth factors, VEGF-C and VEGF-D, and their cognate receptor tyrosine kinase, VEGF

receptor-3 (VEGFR-3), are critical regulators of lymphangiogenesis. Like VEGF-A, VEGF-C stimulates the migration of endothelial cells and increases vascular permeability and endothelial cell proliferation but at higher concentrations than VEGF (8, 10, 18). These signals for endothelial cells are probably mediated through VEGFR-2 in blood vascular endothelial cells, and generally via VEGFR-3 in the lymphatic endothelial cells (8, 10, 18, 58-61). Unlike VEGF, the expression of VEGF-C is not regulated by hypoxia (61). The angiogenic versus lymphangiogenic responses to VEGF-C may depend on the degree of proteolytic processing of its precursor and on the expression of its receptors in the blood versus lymphatic endothelial cells of the target tissue (8, 10, 12, 18, 19). VEGF-C also has synergistic effects with VEGF, during the induction of angiogenesis, and this effect is more prominent in cells expressing both of its receptors (12, 19). In addition, VEGF-C can compete with VEGF in binding to VEGFR-2.

VEGF-D (also known as c-*fos*-induced growth factor or FIGF) (1, 62, 63) shares 61% sequence identity with VEGF-C, and these two growth factors bind to the same receptors on human endothelial cells. VEGF-D has been shown to be able to stimulate the proliferation of endothelial cells, and it shows angiogenic properties *in vitro* and *in vivo* (11). Like VEGF-C, it was also shown to be lymphangiogenic when overexpressed in skin keratinocytes (10).

Skobe and co-workers (56) demonstrated the presence of lymphangiogenesis within the peripheral areas of MDA-MB-435/GFP tumors, suggesting the production of lymphangiogenic factors in this metastatic tumor. Overexpression of VEGF-C resulted in enlargement of peritumoral lymphatic vessels and in increased intratumoral lymphangiogenesis –identifying VEGF-C as a potent tumor lymphangiogenesis factor.

Tumor-associated lymphatic vessels contribute to the efficient transport of fluids and macromolecules and are, therefore, of great importance for overall tumor physiology and drug delivery, but it may not be critical for tumor dissemination (56). Nevertheless, the formation of an intratumoral lymphatic network, whether functional in fluid transport or not, might promote metastatic tumor spread by creating increased opportunities for metastatic tumor cells to leave the primary tumor site.

Skobe and coworkers (56) demonstrated that the increased density of lymphatic vessels within VEGF-C–overexpressing breast cancers was associated with a significantly increased incidence of metastases in regional lymph nodes. Therefore, these findings provide a novel mechanistic insight into the reported correlation of VEGF-C expression in the primary tumors with high incidence of lymph node metastases in, for instance, breast and prostate carcinoma (64). Moreover, a recent study found that VEGF-C

expression was only detectable in node-positive breast cancers, whereas expression of VEGF was detected in both node-positive and node-negative tumors. VEGF-C may promote the incidence of lymphatic metastases, thereby facilitating tumor cell entry into the lymphatics.

In addition to these studies, Kinoshita and co-workers found that VEGF-C may not be directly involved in vascular spread but may act as promising marker to predict recurrence, thus implying that VEGF-C can also play a role in metastatic growth (55).

5. VASCULAR NETWORKS IN OSTEOTROPIC CANCERS AND EXPERIMENTALLY-INDUCED BONE METASTASES.

The dissemination of cancer cells can occur directly into the local tissue, via blood vessels (hematogeneous spread) and lymphatics (lymphatic spread), or by invasion of body cavities (18). Tumor-induced angiogenesis and the expression of various angiogenic factors like VEGF-A, bFGF and their receptors have been identified as prognostic indicators of poor prognosis and disease recurrence.

The lymphangiogenic concept described above is supported by our observations that thin meshworks exist in experimentally-induced bone metastases from MDA-MB-231 cells (Figure 3) (65, 66) and in primary breast carcinomas and their lymph node metastases in patients (Figure 4, upper panels). Experimentally-induced bone metastases from human breast (MDA-MB-231) and prostate cancer (PC-3M-Pro4) in nude mice strongly stimulate angiogenesis (CD31+ blood vessels, Figure 3c) and induce the formation of complex three-dimensional networks (Figure 3d) (65, 66). In these animals, MDA-MB-231 breast cancer cells in mestastatic lesions express a variety of pro-angiogenic factors, including VEGF-A, -B and –C. Strikingly, we found that the expression of VEGF-A, VEGF-B and VEGF-C is strongly and significantly (VEGF-A, -B) upregulated in bone metastases when compared with soft tissue metastases, suggesting that tumor-bone (marrow) interactions play a decisive role in colonization and/or subsequent invasive growth of osteotropic cancers (66). In primary tumors and lymph node metastases in breast cancer patients various Periodic Acid Schiff positive (PAS+) patterns, including PAS+ meshworks, are frequently observed. These PAS+ patterns are often characterized by the lack of Flt-4/VEGFR3 expression, patchy expression of PECAM-1/CD 31 and vimentin, and the continuous expression of Smooth Muscle Actin (SMA).

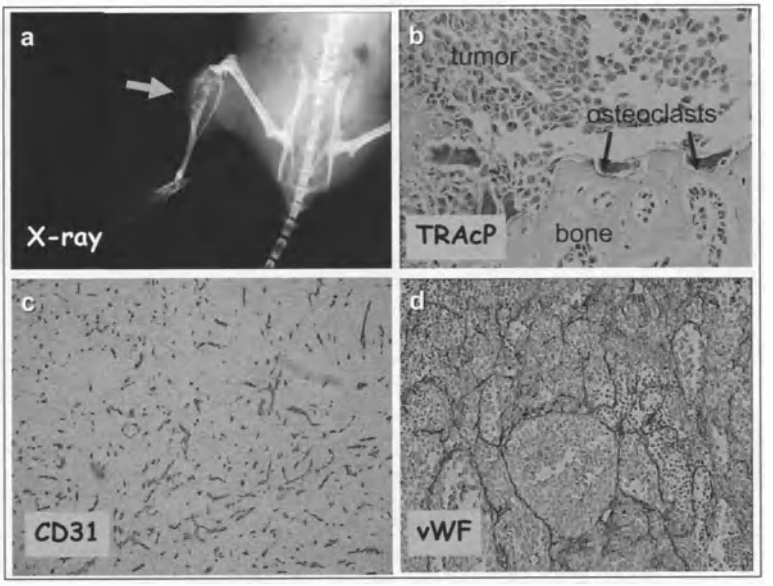

Figure 3. Osteolytic bone metastasis in the tibia of a nude mouse after intracardiac inoculation of 100,000 human MDA-MB-231 breast cancer cells. A. Radiograph showing multiple osteolytic lesion in bone, B. MDA-MB-231 cells have almost completely replaced the bone marrow and strongly induce the formation of Tartrate-Resistant Acid Phosphatase (TRAcP) osteoclasts that resorb surrounding bone, C. Tumor blood caplillaries in a bone lesion stained by the CD31 or PECAM-1 antibody MP-12, D. A section of a bone metastasis stained for von Willebrand factor (vWF) showing a network of interconnected loops.

We have recently found that the prognosis of the patient is inversely correlated with the complexity of the PAS+ meshwork in lymph node metastases (Figure 4, lower panels. Buijs et al., submitted for publication). Furthermore, the presence of a PAS+ network, the most complex PAS+ pattern, in either primary tumor or lymph node metastases from invasive ductal carcinoma of the breast, is one of the three independent factors associated with the recurrence of a distant metastasis and survival (Buijs et al., submitted for publication). The presence of lumina in the PAS+ meshwork might be indicative for septa that could facilitate perfusion and dissemination of cancer cells. Indeed, tumor cells were frequently present in the lumina, whereas, erythrocytes were occasionally found in these structures. In addition, Foss and co-workers provided evidence that PAS+ extracellular septa can be particularly favorable substrates for the in-growth of angiogenic, and possibly lymphatic, vessels (67). Consequently, tumors that contain these PAS+ structures might be more aggressive, as these structures provide better means for (blood) vessel in-growth than tumors

lacking these structures. Alternatively, it cannot be excluded that highly malignant breast cancer cells can acquire an invasive, mesenchymal phenotype, a process called epithelium-to-mesenchyme transition (EMT). It is, therefore, tempting to speculate that tumor cells that aligne hollow tubular structures are phenotypically distinct from other tumor cells. The generation of such a PAS+ network by genetically deregulated, aggressive tumor cells was termed 'vasculogenic mimicry' to emphasize their *de novo* generation without participation of endothelial cells and independent of angiogenesis (68-70). Recent reports suggest that vasculogenic mimicry also exists in ovarian (71), prostate and breast cancer (1, 70, 72-74). After establishing an inflammatory breast cancer (IBC) xenograft with vasculogenic mimicry features, Shirakawa and co-workers examined surgically resected breast cancers and classified 7.9 % as exhibiting vasculogenic mimicry (73). The existence of vasculogenic mimicry increased the likelihood of hematogenous spread and gave a poorer prognosis for the patient.

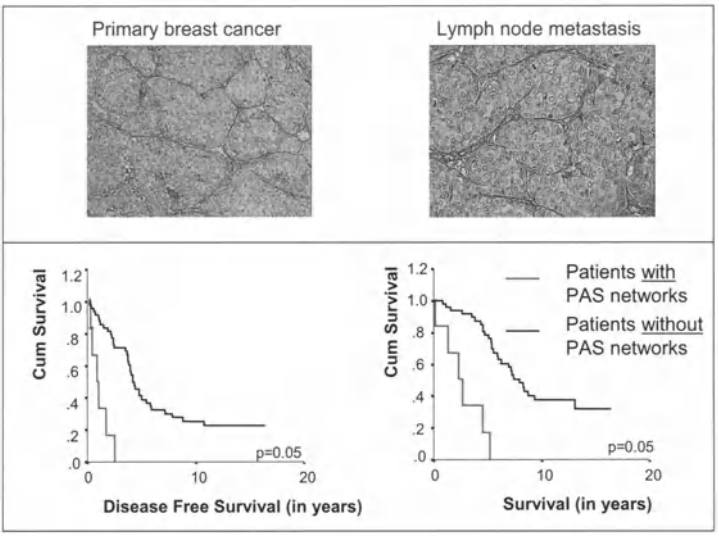

Figure 4. The presence of a periodic acid Schiff (PAS)+ network in the primary tumor or lymph node metastases from invasive ductal carcinoma of the breast (upper panels) is one of the independent factors associated with the recurrence of a distant metastasis and survival (lower panels) as determined by Cumulative disease free survival and overall survival (Buijs et al., submitted for publication).

The lack of continuous Flt4/VEGFR3 expression (VEGF-C receptor and lymphatic marker) in the PAS+ network may indicate that these structures are complex and do not only represent the lymphatic vasculature. If the septa consist of two layers of ECM elaborated by opposing layers of tumor cell,

the layers could separate from another, forming channels and laminar openings. Such openings could particularly lead to capillary growth. The patchy expression of the endothelial marker, CD31/PECAM-1, is in agreement with this hypothesis.

The presence of PAS+ networks, in either primary tumor or lymph node metastases from invasive ductal carcinoma of the breast, is one of the three independent factors associated with the recurrence of a distant metastasis. Moreover, the presence of a PAS+ network in a positive lymph node is the factor most strongly associated with recurrence.

6. OPTICAL IMAGING, A NOVEL NON-INVASIVE TECHNIQUE FOR MONITORING BONE METASTASIS; EVIDENCE FOR AN ANGIOGENIC SWITCH.

Investigation of the pathogenesis of bone metastasis, the involvement of specific genes (functional genomics) and evaluation of therapeutic agents would greatly benefit from a method that could non-invasively image cancer cells *in vivo*. Furthermore, the development of novel anti-cancer and anti-angiogenic strategies requires more sensitive and less invasive methods to detect and monitor *in vivo* minimal residual disease in (breast) cancer metastasis.

Injection of cancer cell lines into the left cardiac ventricle of immunodeficient (*nu/nu*) mice is a widely used animal model of bone/bone marrow metastasis (65, 66, 75-79). This experimental setting closely resembles the clinical situation of minimal residual disease after removal of the primary tumor. However, detection of bone metastasis by radiography does not make it an ideal model of minimal residual disease.

In fact, radiologically evident bone metastases are a late and macroscopic event in the evolution of metastatic bone disease. Thus, the model lacks the sensitivity that would be necessary to dissect the initial processes, such as the *angiogenic switch*, essential for tumor progression. Furthermore, the radiological detection and measurement of skeletal metastases do not represent actual tumor burden. As a result the size of the bone lesions are not only dependent on tumor cell numbers, but also on the ability of the cancer cells to excrete factors that stimulate bone remodeling during the course of the experiment. Only a parallel histomorphometric analysis allows the distinction between a therapeutic effect exerted directly on tumor cells or indirectly through inhibition of bone resorption.

To circumvent these potential problems we have recently established a very sensitive, non-invasive method to detect and monitor directly metastatic growth in bone marrow (and soft tissues) of living animals (80) (Figure 5).

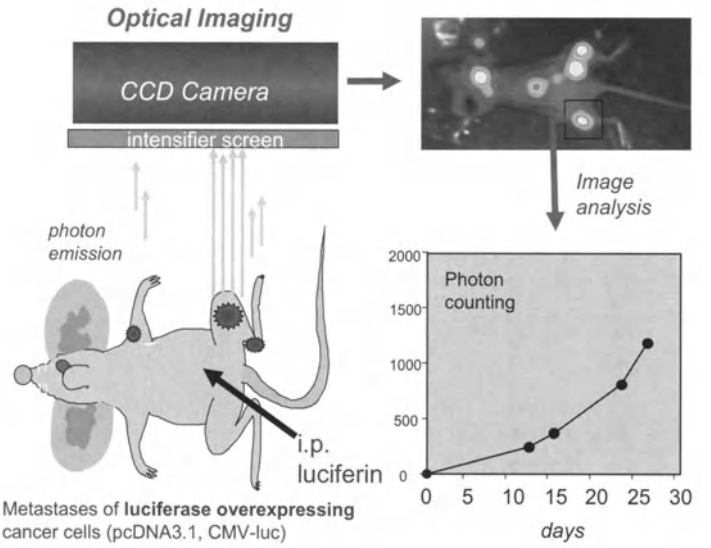

Figure 5. Non-invasive *in vivo* optical imaging of tumor cells that stably express the luciferase gene under a CMV promotor (CMV-luc). After inoculation of cancer cells into the left cardiac ventricle (or directly into the bone marrow, orthotopic sites or subcutaneous tissue of nude mice) the growth of the cancer cells can be monitored upon i.p. administration of luciferin as a substrate for luciferase. Using a very sensitive CCD camera each individual (micro) metastasis can be monitored and their growth can be quantitatively measured (80).

Biological sources of light (bioluminescence) have sufficient intensity to cross animal tissues, provided that the endogenous light has a wavelength between 500 and 1500 nm. Within this wavelength range, the tissues do not absorb photons. The luciferase/luciferin reaction emits photons at wavelengths from 490 to 560 nm and is therefore suitable for external detection. Recent technical advances for imaging weak, visible light sources using charged coupled device (CCD) cameras, peltier-cooled detectors and microplate channel intensifiers allow detection of bioluminescent emission from inside the tissues. Thus, it has been possible to follow in real time the fate of luciferase (*luc*) transfected tumor cells injected in living animals or to monitor gene expression in the live animal via the *luc* reporter gene linked to specific promoters (81, 82). One of the many advantages of this methodology is that it is non-invasive, and therefore allows investigations in the same animal at different time points. Accordingly, the number of the

animal groups in the experimental setting is greatly reduced. This technology is extremely useful in the cancer research area, since it focuses on detection of primary tumor growth, and micro- and macro-metastatic tumor spread.

We have investigated its application as an animal model for bone micro- and macrometastasis by using a *luc*-transfected, bone-seeking sub-clone (MDA-MB-231-B) derived from the human breast carcinoma cell line MDA-MB-231 by four sequential cycles of intracardiac (i.c.) inoculation *in vivo* and expansion *in vitro* of the cell population recovered from the resulting bone metastases (80). We have found the following advantages:

a) early detection of minimal bone metastatic sites (= 0.5 mm^3 volume / 10-20,000 cells);
b) much higher precision and reliability as compared to conventional radiography;
c) the possibility of quantifing the tumor burden for each metastatic site;
d) the possibility of following the kinetics of tumor growth in the same animal;
e) targeted dissection of all the metastatic sites for end-point histological and/or molecular analysis.

Early detection of micrometastases by optical imaging, therefore, also allows monitoring of cancer cells before the angiogenic switch (Figure 6a). This approach can be extremely valuable for the evaluation of new therapeutic agents aimed at repressing initial stages of (metastatic) growth. Using the optical imaging technique we have been able to demonstrate that human breast and prostate cancer cells can initially grow by vessel cooption, but 'switch-on' angiogenesis to allow exponential growth of the cancer cells in the bone marrow microenvironment (Figure 6a).

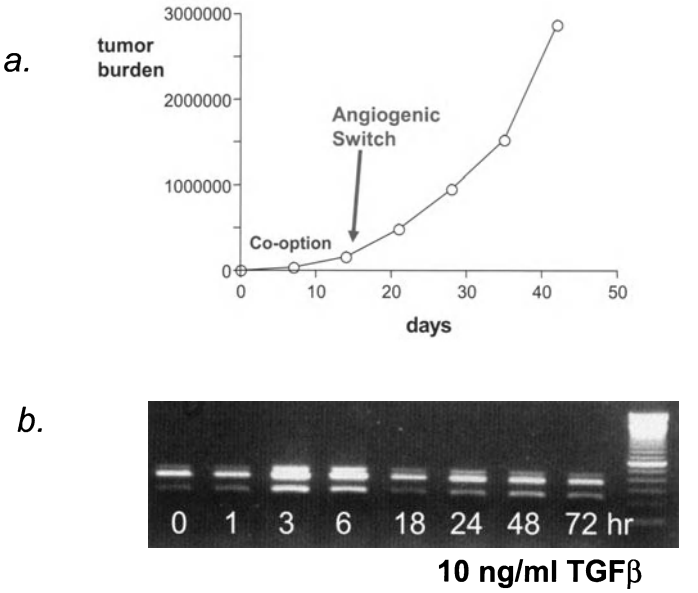

Figure 6. A. Example of the growth of MDA-MB-231B cells (that stably overexpress luciferase) in the bone marrow of nude mice. Initially cells grow relatively slowly (0-14 days), presumably due to cooption of existing blood vessels. Thereafter, the cancer cells grow exponentially, driven –at least in part- by the ability of the cancer cells to stimulate (lymph)angiogenesis, the so-called-angiogenic switch. B. Induction of VEGF-A mRNA expression (bands represent the VEGF121, -165 and –189 splice variants) by MDA-MB-231 cells after stimulation with 10 ng/ml TGF-β at different time points (as determined by semi-quantitative PCR, 66).

7. BONE-DERIVED GROWTH FACTORS AND VASCULAR NETWORKS IN BONE METASTASIS

The TGF-β superfamily, that includes TGF-β and Bone Morphogenetic Proteins (BMPs), is critically involved in bone development, bone remodeling and bone angiogenesis (83-95, 96-98). The bone microenvironment is rich in these soluble factors, particularly since both TGF-β and BMPs are accumulated and stored in the extracellular bone matrix. It is becoming increasingly evident that growth factors like TGF-β, BMPs and IGFs are locally released and activated from the bone matrix by osteoclasts or tumor cells at distinct sites at the bone surface (65, 66, 80, 99-105).

The first demonstration for a crucial role of the TGF-β superfamily in bone metastasis was provided by the study of Yin and co-workers (101). When TGF-β signaling was blocked in breast cancer cells by overexpressing a dominant-negative TRII mutant, immunodeficient mice injected with the cells developed fewer tumors, with less osteoclast recruitment, less bone destruction at metastatic sites and prolonged host survival. The authors provided strong evidence that TGF-β signalling caused increased production of PTHrP by the breast cancer cells, that in turn, stimulated the osteoclastic bone resorption (75, 106). In addition to this self-amplifying process, interactions between cancer cells and bone cells at the bone surface, can lead to enhanced expression of various (lymph)angiogenic factors like VEGF-A, -B and -C in the bone marrow microenvironment by MDA-MB-231 breast cancer cells (66). Interestingly, TGF-β also strongly stimulated the expression of VEGF-A (Figure 6b).

Transcriptional profiling studies in our laboratory have revealed that MDA-MB-231 breast and PC3 prostate cancer cells express a repertory of other pro-angiogenic factors including fibroblast growth factors (FGFs), members of the CCN family (Cyr61, Connective Tissue Growth Factor), some of which are induced by TGF-β and/or BMPs *in vitro* and in bone metastases (van der Pluijm, manucript in preparation).

To date, little is known about the involvement of bone/bone marrow-derived factors like the superfamily (that includes TGF-β and BMPs) and IGFs and their direct involvement in (lymph)angiogenesis, postnatal vasculogenesis and vasculogenic mimicry in metastatic bone disease. It is tempting to speculate that interactions between osteotropic cancer cells and the (a)cellular bone marrow stroma lead to differential expression of (lymph)angiogenic factors and their inhibitors in the bone microenvironment, thus explaining -at least in part- the observed bone tropism of a limited number of carcinomas. These cell-cell and cell-matrix interactions are schematically depicted in Figures 7 and 8.

Taken together, many new tools are now available for further research aimed at studying the interaction between osteotropic cancer cells and the bone marrow microenvironment in bone metastasis and, more importantly perhaps, explore novel therapeutic strategies for treatment of the metastatic bone disease.

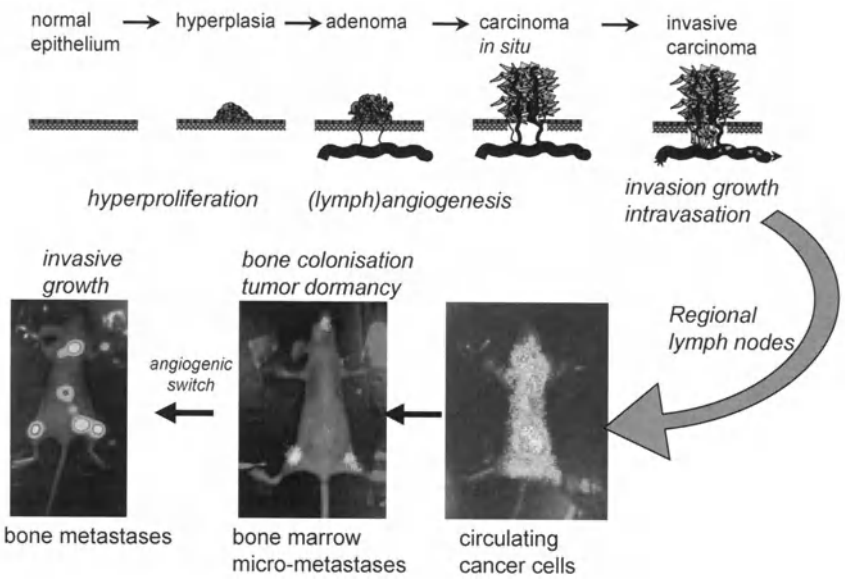

Figure 7. Sequential steps involved in carcinogenesis, dissemination, and metastasis. Carcinomas develop from normal epithelium and undergo sequential genetic changes that, in interaction with the (a) cellular microenvironment, can lead to dissemination and (bone) metastases. The ability of cancer cells to stimulate angiogenesis and lymphangiogenesis at the primary and metastastic sites is of key interest for successful growth and metastasis. Particularly, members of the TGF-β superfamily are involved during carcinogenesis at the primary site and in the devlopment of bone metastasis. Perturbation of TGF-βsignalling cascades in osteotropic cancers may sensitize these tumor cells to growth signals that are intrinsic (or characteristic) to the bone/bone marrow microenvironment, leading to accelerated, invasive growth.

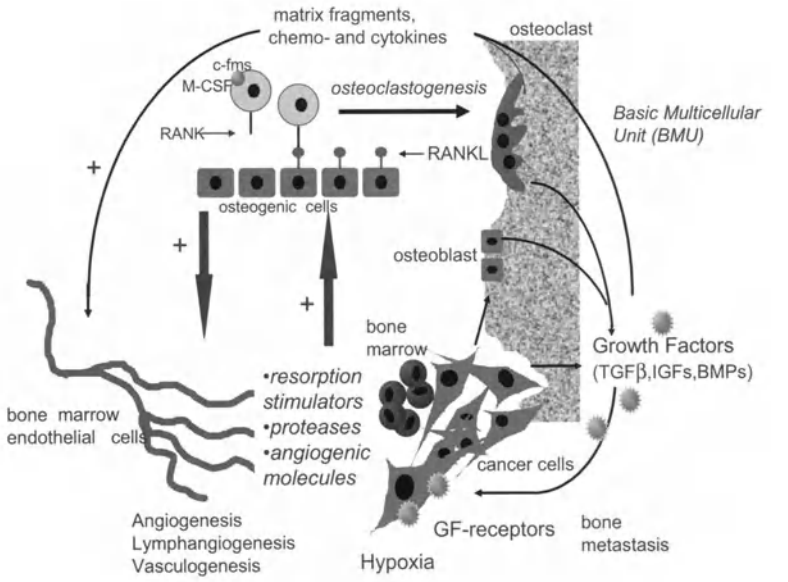

Figure 8. Schematic overview of the vicious cycle of bone metastasis and invasive growth by (breast) cancer cells in bone marrow. Interactions between cancer cells and the bone/bone marrow mivcroenvironment are prerequisites for skeletal metastasis. Soluble factors, including matrix components that are released from the bone surface or produced by supportive bone marrow cells (e.g. TGF-β, IGFs, BMPs) as well as tumor cell - stromal cell interactions are prerequisites for successful colonization and subsequent invasive growth of osteotropic cancers. Acquisition of a microcirculation by angiogenesis, lymphangiogenesis, vasculogenesis and/or vasculogenic mimicry is crucial for the metastatic growth of cancer cells.

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Chapter 9

PROSTATE CANCER: MODELS FOR DEVELOPING NOVEL THERAPEUTIC APPROACHES

Shafaat A. Rabbani[1], Nicholas Shukeir[1], Andrew P. Mazar[2]
[1]McGill University Health Centre, Montreal, Quebec, Canada and [2]Attenuon, LLC, San Diego, California, USA

1. INTRODUCTION

Carcinoma of the prostate is the most commonly diagnosed malignancy among North American males. Currently it is the most commonly diagnosed cancer in men and is the second leading cause of cancer mortality following lung cancer (1-3). Recently, increases in the number of men diagnosed with prostate cancer has been attributed to an increase in the awareness of the disease as well as to better improvements in screening and detection. However, despite this increase in men diagnosed with prostate cancer, early detection and improved treatment modalities for the disease have kept the mortality rate unchanged. There are numerous factors associated with an increased risk of developing prostate cancer although age remains the most prominent risk factor (3-4). Autopsies carried out on deceased males have detected prostate cancer in 50% of men in their 50s with the number jumping to >70% in men over the age of 80 (2). In addition to age, other factors that may contribute to the pathobiology of prostate cancer include hormones, growth factors, diet, vitamins, dietary supplements, environmental factors and viruses (2, 4-15).

Although mortality from prostate cancer has leveled off, prostate cancer nevertheless remains a major health problem for aging men (2). In addition to the potential for mortality, significant morbidity is also associated with the

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disease or treatment of the disease including urinary obstruction, loss of sexual function and psychosocial aspects of the disease in early stage prostate cancer patients' and bone pain, osteoporosis and dementia in advanced prostate cancer patients (16, 17). Thus, prostate cancer remains a serious health problem in most westernized countries and the treatment and earlier detection of prostate cancer continues to remain a challenge. The development of novel diagnostic and interventional approaches requires extensive pre-clinical evaluation in models of prostate cancer before moving into clinical evaluation. This review summarizes recent progress in establishing models of prostate cancer and their utility as tools for the evaluation of various interventional strategies for the treatment and diagnosis of prostate cancer.

2. BIOLOGICAL PROGRESSION OF PROSTATE CANCER

As with other hormone dependent malignancies like breast, prostate and ovarian cancer, the biological progression of prostate cancer is the result of many events that ultimately lead to the development of highly invasive, hormone refractory metastatic prostate cancer. These include genetic mutations causing the inactivation or silencing of tumor suppressive genes (2, 18). Some of the additional factors which facilitate tumor progression include sex steroids (androgens), growth factors, angiogenic factors, proteases and cytokines (1, 19-22).

Encapsulated primary prostate tumors are rarely associated with mortality. Rather, it is the development of tumor metastases that ultimately results in death. In patients with prostate cancer, metastasis to the bone is frequently observed in advanced disease (23, 24). While skeletal metastases can be of the osteolytic or osteoblastic variety, those observed in patients with prostate cancer are usually of the osteoblastic phenotype (25-28). It is believed that these osteoblastic metastases are the result of cancer cells producing factors that stimulate osteoblast proliferation, differentiation and eventually bone formation (25). The process of metastasis in prostate cancer is similar to other cancer types. Aggressive forms of prostate growing in the site of origin can invade their surrounding tissue by producing proteolytic enzymes, such as urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs), which help breakdown the extracellular matrix surrounding the tumor and the basement membrane surrounding tumor-associated blood vessels (ECM) (25,29). Breakdown of the ECM and the basement membrane allows tumor cells to intravasate into the circulation and to disseminate systemically. Cells that reach organs or bone distal to the

prostate can then extravasate from the blood vessels into the stroma of the organ. There they begin growing again with the whole process reinitiated, resulting in the development of tumor metastases at multiple sites.

In addition to proteases, there is increasing evidence to indicate that certain growth factors are involved in the stimulation of bone formation that is associated with metastatic prostate tumors. Patients with prostate cancer have increased levels of endothelin-1 (ET-1), which is known to stimulate bone formation and osteoblast proliferation (26, 27). The TGF-ß superfamily as well as the bone morphogenetic proteins (BMPs), namely BMP-2, BM-3, BMP-4 and BMP-6, have also been shown to be stimulators of bone formation (30). Other growth factors that have been shown to be increased in patients with prostate cancer include parathyroid hormone related peptide (PTHrP), fibroblast growth factor (FGF) and platelet derived growth factors (PDGFs) (31-33). Finally, the amino terminal fragment of uPA has also been shown to exhibit mitogenic activity for osteoblasts, whereas the carboxy terminal proteolytic domain of uPA mediates tumor invasiveness and growth factor activation (34-36). Both of these processes may contribute to the pathogenesis of bone metastasis.

3. ROLE OF GROWTH FACTORS IN PROSTATE CANCER

At least 15-20% of patients with malignancy exhibit hypercalcemia which has now been attributed to the production of parathyroid hormone related peptide (PTHrP) by tumor cells (37, 38). While increased PTHrP production is more commonly observed in patients with breast cancer and multiple myeloma, subsets of prostate cancer patients exhibiting hypercalcemia also show high circulating levels of PTHrP (39). An *in vivo* model of prostate cancer over-expressing PTHrP, where it can act as an autocrine growth factor for tumor cells, was developed to demonstrate the role of PTHrP in promoting prostate tumor growth and to evaluate its ability to promote bone resorbing effects in a malignancy which is more commonly associated with an osteoblastic response (40).

The full-length cDNA encoding rat PTHrP was subcloned into a mammalian expression vector. Dunning R3227 Mat Ly Lu cells were stably transfected with vector alone (CTL) or experimental plasmid (PTHrP) to establish Mat Ly Lu-CTL and MatLyLu-PTHrP cells (40,41). Both control and experimental cells were inoculated s.c. into the right flank of Copenhagen rats and tumor volume was measured at timed intervals. Experimental animals receiving MatLyLu-PTHrP cells were found to have significantly larger tumors when compared to control group of animals

injected with MatLyLu-CTL cells. When these control and experimental cells were injected into the left ventricle of Copenhagen rats, no difference was observed in the time to development of hind limb paralysis. However, analysis of plasma from these animals showed increased levels of calcium and PTHrP in animals injected with cells over-expressing PTHrP. This hypercalcemic response was found to be more pronounced following intracardiac injection as compared to s.c. inoculation of cells, which may be attributed to the established bone resorbing effects of PTHrP when expressed at the site of experimental skeletal metastases (vertebral column). Histological characterization of lumbar vertebrae from control animals showed mixed osteoblastic and osteolytic lesions. However, vertebrae of animals inoculated with cells over-expressing PTHrP exhibited increased osteolytic activity which is attributed to the bone resorbing effects of PTHrP (Figure 1). Despite the lack of effect of PTHrP on bone metastasis progression, the use of the MatLyLu *in vivo* model has allowed the investigation of the growth factor-like effects of PTHrP on tumor growth and has also provided a model that allows the monitoring of biochemical surrogates (e.g., plasma PTHrP and calcium) to establish efficacy when evaluating experimental therapeutics targeting prostate cancer growth (40, 42).

The role of other growth factors and cytokines in the progression of prostate cancer metastasis to bone is also unclear. Lee et al. tested a panel of bone-associated growth factors and cytokines for their ability to stimulate the proliferation of prostate cancer cell lines isolated from both soft tissue as well as osseous metastases (43). Variable effects were observed with no clear differentiation in response between the tumor lines isolated from soft tissue compared to those isolated from bone metastases. The role of angiogenesis in promoting the metastastic potential of prostate cancer cells has been well elucidated in other cancer types and may be involved in prostate cancer metastasis as well. The onset of angiogenesis is the result of a change in the equilibrium between pro-angiogenic and anti-angiogenic molecules (44). Several pro-angiogenic molecules include VEGF, FGF and PDGF have been implicated in prostate cancer progression (45, 46). PDGF may play an especially important role in the progression of prostate cancer metastasis as demonstrated in a recent study by Uehara et al. (47).

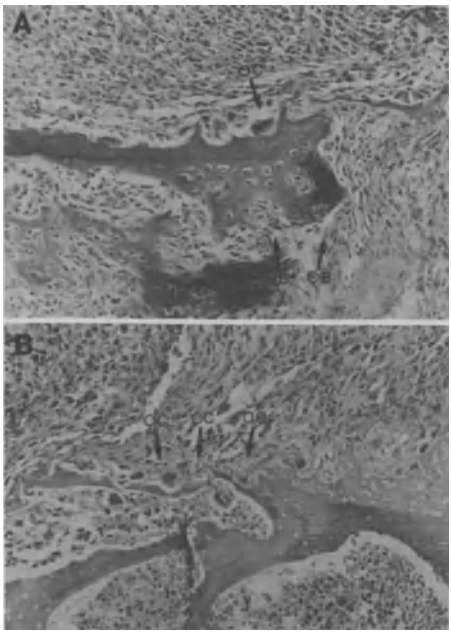

Figure 1. Effect of PTHrP over-expression on skeletal metastases. Male Copenhagen rats were inoculated with MatLyLu (panel A) and MatLyLu-PTHrP cells via intracardiac route of inoculation. At the time of development of hind limb paralysis, animals were sacrificed and histological analysis of lumbar vertebrae from control and experimental animals was carried out. Arrows indicate old bone (OB) and clusters of multinucleated osteoclasts (OC) in areas of bone resorption of experimental animals.

In this study, male athymic mice were injected intra-tibially with a PC-3 cell sub-line (PC-3MM2). Starting at day 3 post tumor cell inoculation, animals were assigned to receive one of four treatment protocols

a) daily dose of vehicle alone;
b) weekly dose of paclitaxel;
c) a daily dose of a known inhibitor of the PDGF receptor, STI 571 (Gleevec) and;
d) a combination of both a weekly dose of paclitaxel and a daily dose of ST1571.

Tumor size and osteolysis of the injected bone were evaluated by digital radiology at the end of the study (47). All of the control animals inoculated with the PC-3MM2 cells and receiving vehicle alone developed osteolytic lesions. While administration of paclitaxel alone had no significant effect on bone lesions, administration of ST1571 alone resulted in a 50% reduction in the number of animals developing osteolytic lesions. Furthermore, the

average tumor weight of the lesions in animals receiving either paclitaxel or ST1571 was substantially lower than in control animals receiving vehicle alone (47). The combination of both paclitaxel and ST1571 was the most efficacious treatment in this study, leading to the largest reduction of osteolytic lesions and the lowest tumor weights of all the treatment groups (47). This study implicates the PDGF pathway in the formation and progression of prostate cancer metastasis to bone and provides a validated target that can be evaluated therapeutically in the clinic. This study also emphasizes the probable need for combination therapy approaches for the successful treatment of bone metastasis in patients.

3.1 Role of Proteases in Prostate Cancer

Urikinase Plasminogen Activator (uPA), a member of the serine protease family, is expressed as an enzymatically inactive single chain pro-enzyme (48). Following its activation to the two-chain, enzymatically active form which occurs through limited proteolysis, uPA can activate plasminogen to plasmin, initiating a cascade of events that leads to the activation of several matrix metalloproteases and the release of latent growth factors sequestered to the ECM. Collectively, this chain of events plays a major role in tumor progression. Additionally, the uPA cascade also leads to the degradation and remodeling of the tumor ECM through its effects on laminin, fibronectin, fibrin and collagen (36, 48). This facilitates the intravasation of tumor cells into blood vessels and metastases to distant organs. Finally, uPA plays a major role in promoting the invasive and metastic behaviour of several malignances by directly promoting cell growth, migration and angiogenesis, inducing signals mediated by its specific cell-surface receptor, uPAR (49).

In order to directly establish the role of uPA in prostate cancer progression and metastasis, a syngeneic model of rat prostate cancer was established. MatLyLu rat prostatic carcinoma cells were transfected using experimental plasmids containing rat uPA in both sense and anti-sense orientations (35). This model was established to overcome the issue of species specificity as human uPA fails to bind effectively to rat uPAR and vice versa. Since uPA and uPAR are expressed not only by tumor cells but also by angiogenic endothelial cells and tumor associated macrophages, only a syngeneic or allogeneic model would allow for the full complement of possible uPA-uPAR interactions within a tumor to occur. Control groups of rats inoculated in the left ventricle with untransfected MatLyLu cells routinely developed hind limb paralysis by day 21 post inoculation (35). In contrast, animals receiving Mat Ly Lu cells transfected with the sense uPA vector (MatLyLu-uPA-S) developed paralysis by day 14 whereas the animals inoculated with the anti-sense uPA MatLyLu cells (MatLyLu-uPA-

AS), which expressed lower levels of uPA than the MatLyLu cells transfected with vector alone (MatLyLu- PYN), did not develop hind limb paralysis until day 27 (Figure 2).

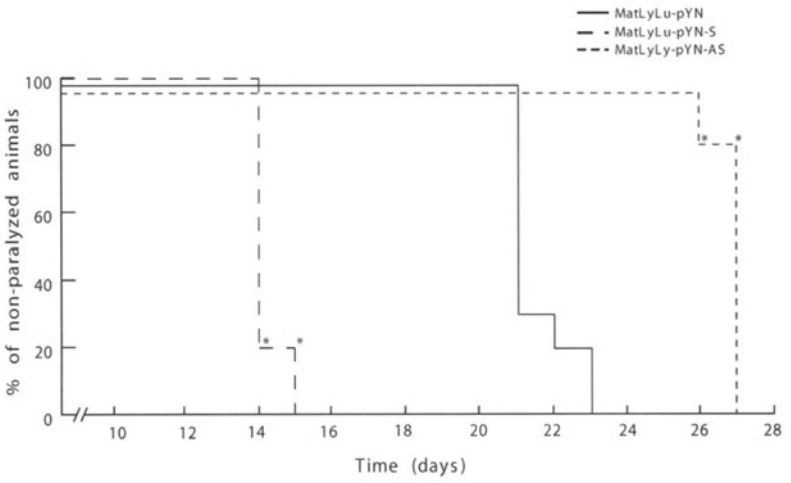

Figure 2. Effect of uPA production on the development of hind limb paralysis. Male Copenhagen rats were inoculated with MatLyLu cells transfected with vector alone (MatLyLu-PYN) or uPA cDNA in anti-sense (MatLyLu-YN-uPA-AS) or sense (MatLyLu-PYN-uPA-S) orientation. The percentage of animals developing hind limb paralysis in each group at various time points was compared. Significant differences from control (MatLyLu-PYN) is shown by asterisks ($P<0.05$).

Our previous studies had shown that uPA as well as its catalytically inactive amino-terminal fragment were mitogenic for cells of the osteoblastic phenotype *in vitro* and the MatLyLu model allowed us to evaluate the ability of uPA to act as selective mitogen for cells of the osteoblastic phenotype *in vivo* in the context of bone metastasis progression (35). Rats inoculated with parental MatLyLu cells commonly exhibited both osteoblastic and osteolytic lesions. However, animals inoculated with MatLyLu-uPA-S (uPA over-expressing) cells demonstrated a predominantly osteoblastic response in their bone metastases (observed using histological analysis and measurement of serum alkaline phosphatase), effects that were attributed to the mitogenic effects of uPA for cells of the osteoblastic phenotype (Figure 3A). In addition, the serum from control and experimental animals was tested for any change in biochemical parameters associated with increased bone turnover. Experimental animals receiving MatLyLu-uPA-S cells showed increased levels of serum alkaline phosphatase compared to the control group of animals inoculated with MatLyLu-PYN cells (Figure 3B).

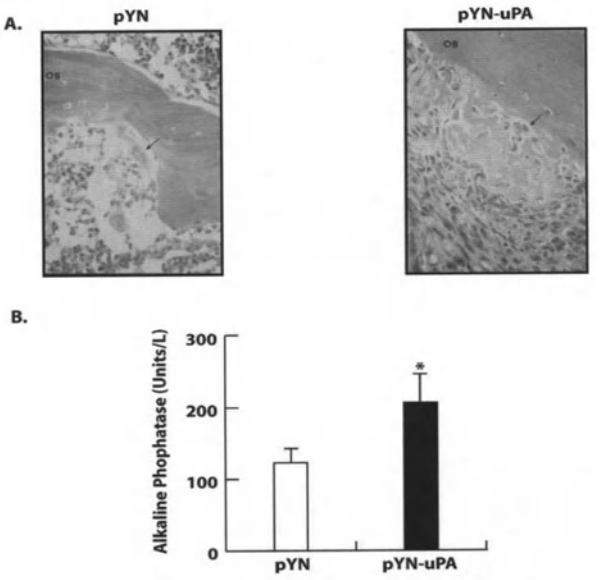

Figure 3. Effect of uPA production on bone turnover. **A**: Light micrograph of lumbar vertebrae of animals inoculated with MatLyLu cells transfected with vector alone (PYN) or cDNA encoding uPA in sense orientation (PYN-uPA). Areas of old bone (OB) and multinucleated osteoclasts in control (PYN) and incrased osteoblastic activity in (PYN-uPA) each group of animals is indicated. **B**: Serum from control (PYN) and experimental (PYN-uPA) animals was analyzed for the level of alkaline phosphatase at the time of developing hind limb paralysis. Significant difference from control is shown by asterisks ($P<0.05$).

Detailed analysis of control and experimental animals also showed that control MatLyLu-PYN animals developed tumor metastases to adrenals, kidney, lungs in addition to skeletal metastases to the lumbar vertebra. Animals receiving MatLyLu-uPA-S routinely developed significantly greater numbers and size of tumor metastases to soft tissues. Additionally these animals not only developed metastases at lumbar vertebrae L1-L2 at a much earlier time period than control animals but also exhibited the presence of skeletal metastases at additional sites (scapula, femur and ribs) (Table 1). Collectively, these studies provide evidence for the role of uPA in tumor cell colonization to skeleton as well as a model for the evaluation of therapeutic strategies targeting the uPA system (35, 36).

Table 1. Assessment of tumor metastases in control and experimental animals. At the time of sacrifice male Copenhagen rats receiving MatLyLu cells transfected with vector alone (MatLyLu-PYN) or uPA (MatLyLu-uPA). Total number of macroscopic metastatic foci at different sites in each group is indicated.

Site of Tumor Metastases	MatLyLu-PYN	MatLyLu-PYN-uPA
Adrenals	2	4
Kidneys	1	3
Lungs	1	2
Vertebral Column	2	4
Scapula	-	2
Ribs	-	2
Femur	-	1

4. MODELS OF PROSTATE CANCER

In order to establish the role of various growth factors and proteases in the multi-step process of prostate cancer progression as well as to evaluate experimental therapeutics, suitable *in vivo* models are needed which best mimic the natural behavior of human prostate cancer. Although this has been a major objective in prostate cancer research for many years and the issue of suitable models has been addressed in various workgroups and publications, the search for suitable models of prostate cancer progression continues (50-52). Human prostate cancer starts as a local non-invasive androgen sensitive disease that may be indolent for some amount of time and may slowly progress into highly invasive, hormone insensitive disease. This progression has been difficult to model in any one single or combination of models. Thus, while several *in vivo* models of prostate cancer are available, continued efforts are needed to develop representative models of human disease. In these models, one would desire reproducibility and progression of disease including invasive behavior and vascular and lymphatic spread to distant organs including bone, where tumor cells can effectively proliferate in the microenvironment of the bone. This is particularly important since tumor or bone derived factors can interact co-operatively in an autocrine or paracrine manner to progressively promote local tumor growth as well as tumor invasion and metastasis to unaffected sites as is often seen in human prostate cancer over time. Prostate cancer can also develop spontaneously in dogs, cats, rats and transgenic mice, which can serve as suitable models for study of this disease. However, these animal models do not develop metastasis and thus, the study of metastasis has been restricted to artificial experimental systems.

4.1 Transgenic Models

While several transplantable models of prostate cancer are commonly used for evaluating experimental therapeutics *in vivo*, autochthonous models have also contributed significantly to our understanding of prostate cancer pathobiology. Use of transgenic and knock out technology in these models has provided a unique opportunity to investigate the significance of a particular gene in the complex process of tumor initiation and progression (53). The transgenic adenocarcinoma of the mouse prostate (TRAMP) has been used effectively to study the role of peptide growth factors in prostate cancer (54, 55). In this well-characterized system, several important features of prostate cancer have been observed including progression from hormone dependence to hormone independence and metastasis to distal organs. This model also allowed investigators to obtain genetic and biochemical information on prostate cancer progression while continuing to maintain an intact immune system. Unfortunately, the TRAMP model is limited due to a lack of representative markers of prostate cancer progression in mice. For example, mice do not express prostate specific antigen (PSA), which makes it difficult to follow disease progression in this host. However, with emerging technologies such as inducible knock-in and knock-out mice, continued progress is being made to develop models that more accurately reproduce human prostate cancer (56, 57). These inducible gene expression systems combined with new state of the art imaging technologies will provide better insight into the development and progression of this disease. Ultimately, newly developed models may become the gold standard for evaluating novel prostate cancer therapeutics but until then, the study of prostate cancer progression and the evaluation of new drugs with the potential for treating prostate cancer will continue to be carried out using existing transplantable prostate cancer models. We will therefore discuss the most commonly used syngeneic and xenograft models of prostate cancer and their utility for studying mechanisms of prostate cancer progression as well as for evaluating novel therapeutic approaches.

4.2 Syngeneic Models of Prostate Cancer

Syngeneic models have several advantages over xenograft models of cancer: there are no species barriers to overcome, since both tumor line and host are derived from the same species; the host is immunocompetent; and tumor take rate and growth are extremely efficient since the tumor line is put back into the same strain of animal from which it was derived, making rejection unlikely. Indeed, numerous tumor lines have been developed from syngeneic systems. For prostate cancer, one of the most frequently used

model systems is the Dunning R3227 rat prostate cancer cell line (41). This line was originally identified as a spontaneous adenocarcinoma of the prostate and was found to be slow growing and non-metastatic. However, the isolation of the parental R3227 cell line has led to the establishment of numerous sub-clones with various properties (41). These include the slow-growing, hormone sensitive, androgen receptor positive cell line R3227H and the hormone insensitive, androgen receptor negative, highly invasive R3227AT cell lines. Due to the ability of the R3227AT cells to metastasize to lymph nodes and lungs when implanted into syngeneic hosts (male Copenhagen rats), this line is also referred to as Dunning R3227 – Mat Ly Lu rat prostate cancer cell line (58-61). Since these cell lines represent different stages of prostate cancer progression (from hormone sensitive to hormone insensitive), they have frequently been used to assess the contribution of a gene of interest to the development of prostate cancer as well as for evaluating anti-tumor response to hormonal and bio-therapeutic agents. Apart from the Dunning R3227 parental and sub-cloned cell lines, other less frequently used models of rat prostate cancer including the Lobund-Wistar (L-W) rat and the Wistar-Unilever (WU) rat have been developed (62-65). In the L-W rat model, animals develop spontaneous tumors in the anterior prostate, possibly due to high circulating levels of testosterone. Early stage tumors are hormone dependent for their growth but do become hormone independent as the tumors progress and ~30% of animals will develop metastatic disease (66, 67). This model has been used to test the effect of various interventions on prostate cancer development and has demonstrated the inhibitory effect of diverse approaches such as dietary testosterone ablation, tamoxifen and vitamin D analogues on prostate cancer growth (68, 69). Unfortunately, similar to transgenic models of prostate cancer, the L-W rat is not practical for testing drugs with potential activity against prostate cancer. The spontaneous tumors take a long time to form and it takes ~25 months to develop the most aggressive form of the disease. It would also be difficult to evaluate the effect of a drug on survival in this model since the animals that develop aggressive disease are already very old and will most likely die from something other than cancer before an effect on survival could be observed. The WU model is a hormone dependant prostate cancer model that does not progress to metastatic or hormone insensitive disease and really lends itself to chemoprevention and tumorigenesis studies only. Cell lines developed from L-W rats include PAIII. These cells, when injected close to bone, have been reported to cause a mixed osteoblastic and osteolytic response (70).

MatLyLu cells have been particularly useful for target validation and therapeutic intervention studies (35, 40, 42). Following subcutaneous inoculation of these tumor cells into Copenhagen rats, palpable primary

tumors develop by day seven post tumor cell inoculation. These tumors continue to grow in size and lead to tumor metastasis to lungs and lymph nodes which can be readily detected and quantitated by approximately day 18-20, when these animals are sacrificed. Since its development, various Dunning R3227 models of rat prostate cancer have been extensively used for the development and validation of new therapeutic approaches for hormone responsive and hormone refractory prostate cancer. A lytic peptide was tested for its capacity to reduce primary tumor growth and metastases. This peptide was found to be an effective anti-tumor agent in both androgen receptor positive (G) and negative (MatLyLu) Dunning R3227 cells without exhibiting any noticeable side effects on tumor bearing animals (58-60, 71). In another orientation of this model, it is possible to establish experimental metastases to bone by injection of tumor cells into the left ventricle of a rat. These metastases continue to grow in size over the period of 2-3 weeks, eventually causing spinal cord compression and hind limb paralysis (35). The endpoint of this model is easily observed when the animals begin to drag their hind limbs and gives a readout of drug efficacy even before animals are euthanized. Autopsy of these animals also routinely shows distended urinary bladders as well as occasional metastasis to the adrenal glands. Additional details of the experimental metastasis model using Mat Ly Lu cells are discussed in the section titled Role of Proteases in Prostate Cancer.

Production of tumor derived factors at the site of bone metastases has a direct impact on bone turnover. These factors can either act directly or by their ability to activate latent growth factors. These effects are localized within the tumor or bone derived cells where selective receptors for these factors are expressed to direct osteoblastic or ostelytic response. Notable among these are uPA with selective mitogenic effects for osteoblasts, localization of ET-1 due to expression of its receptor on osteoblasts and osteoblast specific transcription factor *Cfba1* which has been implicated in osteoblast differentiation during development and also in bone formation (72, 73).

4.3 Xenograft Models of Prostate Cancer

Although syngeneic animal models provide an excellent opportunity for the evaluation of potential novel therapeutic modalities for prostate cancer, representative models of human origin that mimic the clinical process of the disease are essential to validate these new concepts for potential testing in humans. The introduction of immunodeficient mice such as nude and scid as hosts for xenograft implantation of human prostate cancer opened a new era of cancer research. Unfortunately, the limited availability of experimental

model systems has hampered human prostate cancer research for quite a number of years. The lack of representative models was due to the poor growth potential of the implanted human prostate cancer cells/tissues as well as the fact that many of the early prostate cancer cell lines (PC-3, LNCaP, DU145) lacked many of the features of human prostate cancer progression (74). However, in recent years, additional human prostate cancer lines more closely resembling the characteristics found in human prostate cancer have been identified and established. Tumor lines such as CWR22 and CWR22R, LuCaP, RC-9, MDA, VCaP and C4-2 have demonstrated improved tumor takes, spontaneous metastasis and have become increasingly more prominent in the study of prostate cancer progression as well as in the evaluation of novel interventional strategies (75-78). To date, approximately 25 xenograft models of prostate cancer have been established. The available xenograft models represent various stages of the disease ranging from early stage, non-invasive prostate cancer to late stage, highly invasive prostate cancer.

Early stage, well encapsulated prostatic tumors are curable by surgical removal of the tumor mass (79). However the majority of patients diagnosed with prostate cancer have some degree of soft tissue and skeletal metastases which ultimately leads to the high degree of mortality seen among prostate cancer patients (80). Due to the limited therapeutic modalities available to combat this disease at its hormone independent, highly invasive state, sublines of the human prostate cancer PC-3 cells have been used to develop and validate appropriate therapeutic modalities. Several methods of inoculation have been reported and they include s.c., i.c. and orthotopic injections into the long bones or the prostate gland itself. Several human prostate cancer cells lines can also be injected subcutaneously into the right flank of male nude mice which results in the growth of primary tumors that grow and develop systemic metastases to soft tissues such as lungs (81). Although very convenient for the measurement of primary tumors, this model has a major disadvantage of having a high degree of irreproducibility. A more reliable and highly reproducible method involves the orthotopic injection of prostate cancer cells directly into the prostate of mouse (82). This model has been shown to result in rapid local tumor growth with a high rate of soft tissue metastases.

Intracardiac injection of human prostate cancer cells into the left ventricle of nude mice results in bone metastases. This model has many advantages including the testing of Paget's "seed and soil" theory where the cancer cells serve as the "seeds" and the bone microenvironment serves as the "soil" (83, 84). One of the disadvantages of using such a system is the uncertainty in the pathogenesis of the resulting bone metastases after the cells have been injected into the left ventricle. The metastases occur at sites of active bone remodeling, in young mice, with the majority of metastases occurring at the

metaphysis of the long bones due to the high number of blood vessels at these sites. The unique anatomical arrangement of the blood vessels in this region provides for common embolism sites (e.g., sites where the injected tumor cells will aggregate) leading to the development of bone metastases in growing rodents.

Human prostate cancer cells can also be injected into the tibia of nude mice. Although more recently developed and established, the many advantages that this mode of tumor cell inoculation possesses has established this mode of transplantation as the preferred method for many bone metastases studies. Other than the relative ease of inoculation compared to i.c. injection, several investigators have reported high success rates when injecting PC-3 cells into the tibia of nude mice. Furthermore, the tumors that develop within the tibia are radiologically and histologically similar to those encountered clinically. Unfortunately, due to the experimentally induced nature of the bone metastases, this method cannot be used to study the early metastatic events such as migration, intravasation and extravasation.

During the complex multistep process of tumor progression, prostate cancer is initiated as an androgen-sensitive cancer followed by gradual transition into a highly metastatic and androgen-independent disease that lacks the expression of functional androgen receptors (AR) (85). Studies were carried out evaluating the effect of re-establishing the androgen sensitivity of late stage prostate cancer. Full length cDNA encoding human AR was subcloned into a mammalian expression vector and transfected into human prostate cancer PC-3 cells (PC-3T). Both control PC-3 cells and experimental PC-3T cells were injected subcutaneously into the right flank of male Balb/c (*nu/nu*) mice and animals were monitored for the development of tumors. Control PC-3 animals developed palpable tumors by week 6 post tumor cell inoculation. In contrast to this, there was a significant delay in tumor growth in animals inoculated with androgen sensitive PC-3T cells and palpable tumors were not observed until week 12 post tumor cell inoculation (86). Furthermore, when PC-3T cells were injected into castrated male Balb/c (*nu/nu*) mice, palpable tumors were observed at a much earlier time compared to the non-castrated animals receiving PC-3T cells. In addition, experimental animals inoculated with PC-3T cells exhibited minimal tumor metastases to the lungs as compared to control animals inoculated with parental PC-3 cells (Figure 4). Analysis of tumoral RNA from control and experimental animals showed decreased levels of PTHrP mRNA in PC-3T tumors due to androgen mediated downregulate of PTHrP expression (86). Collectively, these studies demonstrated the ability of sex steroids to regulate the production of growth factors like PTHrP and confirm the role of androgens in prostate cancer progression *in vivo*.

As mentioned previously, the major cause of mortality from prostate cancer is the metastasis of hormone-refractory cells, especially to bone. Several therapeutic modalities targeting bone metastasis have failed to demonstrate regression in the clinical setting (although a decrease in skeletal-related events and palliation of bone pain was observed in a recently published randomized study of Zoledronate (87). Thus, the identification of effective treatments targeting prostate cancer bone metastasis remains a major unmet clinical need.

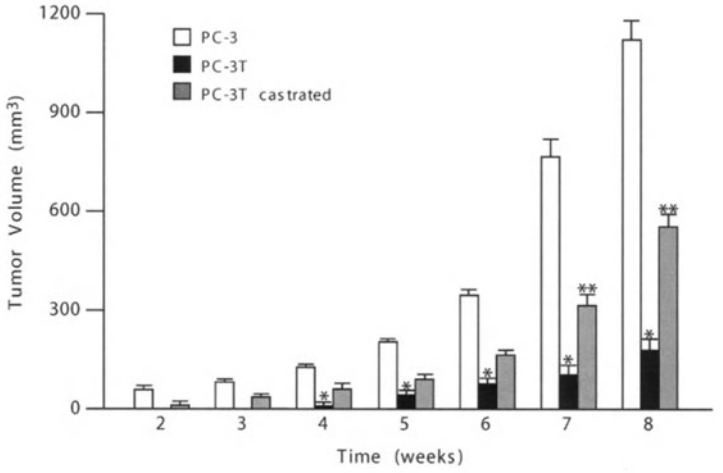

Figure 4. Effect of androgen on prostate tumor cell growth *in vivo*. Human prostate cancer cells PC-3 and cells transfected with androgen receptor (PC-3T) were inoculated into the right flank of normal or castrated male Balb/c mice. Tumor volume was measured at regular intervals. Significant differences in tumor volume from control PC-3 tumor bearing animals is shown by asterisks ($P<0.05$).

4.4 Spontaneous Models of Prostate Cancer in Dogs

Spontaneous prostate cancer in dogs represents a model system that very closely approximates the progression of prostate cancer in man. Prostate cancer occurs most often in elderly, sexually intact dogs or dogs that underwent castration after sexual maturity (88). Most dogs develop adenocarcinoma although the primary tumors tend to appear histologically heterogeneous (89). In a retrospective study of 76 randomly selected dogs with prostate cancer, 80% had metastasis and 22% had metastasis to the bone (88). Those dogs that had bone metastasis demonstrated a predominantly axial skeletal distribution of lesions which is very similar to

the distribution of metastases in men with prostate cancer. Therefore, dogs with prostate cancer may offer insight into the progression of prostate cancer in man as well as a system where promising novel therapeutics could be evaluated.

5. EFFICACY OF PROTEASE INHIBITORS IN *IN VIVO* MODELS

Among the various proteases, MMPs are known to play a significant role in the progression of several malignancies including prostate cancer. Thus, the MMP inhibitor A-177430 was evaluated in the MatLyLu model of rat prostate cancer. This new class of MMP inhibitors showed high activity against several key members of MMP family including MMP2 and 9 (90). In Matrigel invasion assays, A-177430 inhibited the invasion of MatLyLu cells through Matrigel in a dose-dependent manner. *In vivo,* A-177430 was infused twice daily at different doses (10 -100 mg/kg) for 15 days following s.c. inoculation of 1 X 10^6 MatLyLu cells. Primary tumor volumes were obtained at timed intervals and animals were sacrificed and evaluated for the presence of macroscopic tumor metastases at day 16 post tumor cell inoculation. These studies demonstrated a dose-dependent effect of A-177430 on reducing primary tumor growth (Figure 5). In addition, while control groups of animals routinely showed large numbers of metastatic tumor foci at lungs, lymph nodes and kidneys, administration of the highest dose of A-177430 (100 mg/kg/day) showed a marked reduction and in some cases, a complete absence of tumor metastases especially in the lungs of experimental animals. The anti-tumor effects of A-177430 were attributed at least partially to the ability of this agent to promote tumor cell apoptosis (Figure 6).

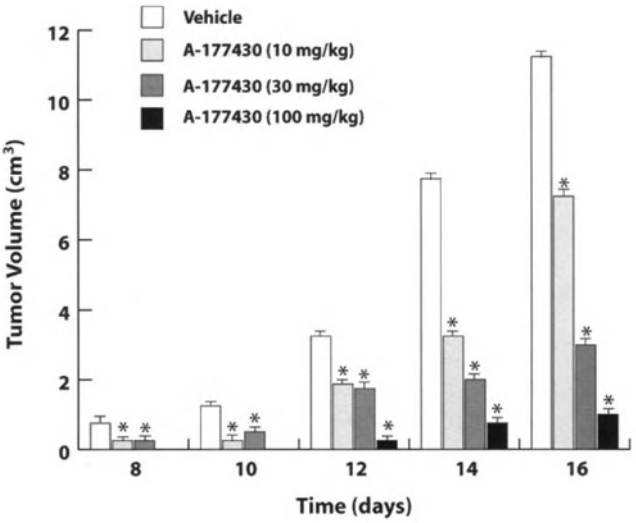

Figure 5. Effect of MMP inhibitor (A-177430) on MatLyLu tumor volume. Male Copenhagen rats inoculated MatLyLu cells (1 X 10^6) into the right flank were treated with vehicle alone or different doses of A-177430. Tumor volume was measured at timed intervals. Significant difference from control is shown by asterisks ($P<0.05$).

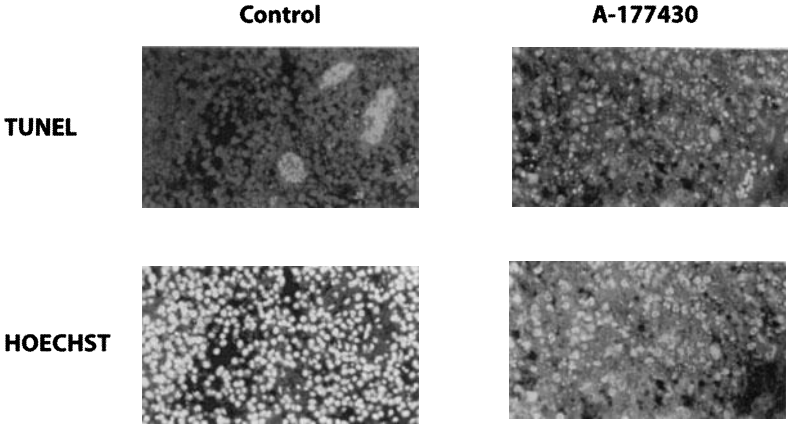

Figure 6. Effect of A-177430 on MatLyLu tumor cell apoptosis *in vivo*. Primary tumors from animals inoculated with MatLyLu cells receiving vehicle alone or A-177430 (100 mg/kg/day) were removed. Following paraffin embedding, sections were processed by TUNEL assay or after TUNEL assay counterstained with Hoechst solution.

The efficacy of a selective inhibitor of uPA (B-428) has also been evaluated in this model. The syngeneic MatLyLu model has been particularly useful in evaluating inhibitors of the uPA system since rat uPA produced by tumor cells was able to fully interact with uPAR expressed by tumor cells and tumor surrounding stromal cells (in contrast to xenograft models, where a species barrier exists to the binding of human uPA to mouse uPAR and vice versa) (91). In addition, the availability of uPA overexpressing tumor cells (MatLyLu-uPA-S) has been useful in establishing highly aggressive models of metastasis. S.c. inoculation of MatLyLu-PYN and MatLyLu-uPA-S cells into Copenhagen rats showed increased tumor growth and metastases in animals receiving MatLyLu-uPA-S. Following s.c. inoculation of these tumor cells, animals were infused with vehicle alone or B-428 (0.24-2.40 mg/kg/day) by implantation of osmotic mini-pumps and tumor volumes were measured at timed intervals. Animals treated with B-428 showed a dose dependent decrease in tumor volume than control group of animals receiving vehicle alone (Figure 7). Finally, a recent study demonstrated that the uPA inhibitor, maspin, could suppress tumor growth, osteolysis and angiogenesis when maspin-overexpressing DU145 cells were injected into human fetal bone fragments implanted in immunodeficient mice (92). Collectively, these results illustrate the central role of the uPA system in prostate cancer progression and validate this system as a therapeutic target for the treatment of prostate cancer.

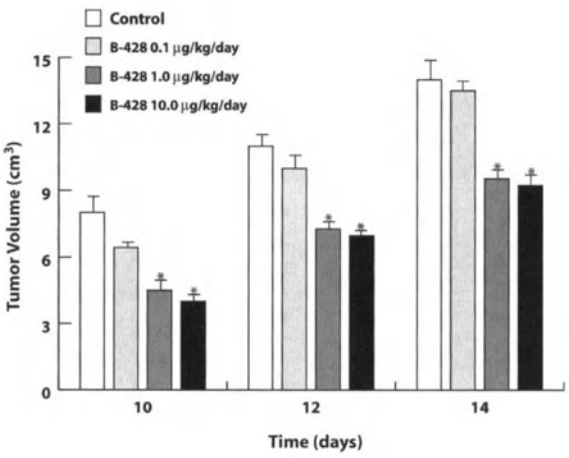

Figure 7. Effect of B-428 on tumor volume. Male Copenhagen rats were inoculated with MatLyLu cells into their right flank. Animals were treated with vehicle alone or B-428 and tumor volume measured at timed intervals. Significant differences from control are shown by asterisks ($P<0.05$).

6. SUMMARY AND CONCLUSIONS

In the past decade, significant improvements and advances have been made in the way prostate cancer is studied in model systems. The advent of novel transgenic and spontaneous models, as well as the establishment of novel cell lines from both spontaneous models and from men with prostate cancer have improved our understanding of the mechanisms responsible for prostate cancer progression. These same models have been used to identify and evaluate novel therapeutic and diagnostic approaches and many of these have been translated into clinical testing. Although many challenges remain, novel high throughput technologies utilizing genomic and proteomic approaches are rapidly changing the way the medical community diagnoses and treats prostate cancer and many of these discoveries are being implemented to more accurately model prostate cancer progression in animals. These improvements will increase our ability to understand prostate cancer progression and ultimately will lead to the development of improved therapeutics for use in this patient population.

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Chapter 10

POTENTIAL THERAPEUTIC TARGETS FOR BONE METASTASIS

Sadmeet Singh and Gurmit Singh
Juravinski Cancer Centre and McMaster University Ontario, Hamilton, Ontario, Canada

1. INTRODUTION

The mortality and morbidity caused by cancer is largely a result of distant spread of the disease. Certain malignancies have a particular propensity to spread to bone amongst which are the three most common cancers – lung, breast and prostate. Patients with lung cancer are more likely to succumb quickly to their disease than those with breast or prostate tumors in whom, relatively, bone metastases are more of a problem. Patients with advanced breast and prostate cancers usually develop bone metastases and tend to harbour the bulk of their tumor burden in their bones at the time of death. The concepts and data reviewed in this chapter relate mainly to these malignancies although some are also relevant to bone metastases secondary to multiple myeloma.

The understanding of molecular mechanisms underlying the pathophysiology of bone metastases is advancing rapidly and as a consequence different processes and steps of the metastatic cascade are being revealed as potential targets for therapy. Increasing numbers of targets are also being identified as a result of the powerful genomic and more recently proteomic screening strategies being employed.

Amongst the phenotypic characteristics of tumors that metastasize to bone are the expression of homing receptors, their associated signaling molecules, extracellular matrix-degrading proteases and other factors responsible for destruction of bone. The influence of the bone environment has long been recognized as a crucial facilitator of bone metastasis and again

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as the mechanisms involved are unravelled, further opportunities for therapeutic intervention arise.

When considering genetic targets for metastasis treatment it is noteworthy that many of the genes classically recognised as oncogenes are affected by point mutations, deletions or translocations that can make targeting them for therapy problematic. Genes involved in cancer spread are not usually mutated in the same way, which is advantageous when considering novel therapies. The genetic stability offered by such genes and those of the normal bone stroma as a therapeutic target makes them unlikely to develop modifications rendering them resistant to therapy.

Although the pathophysiology of bone metastasis and certain therapeutic targets are covered in depth in other chapters, this chapter provides an overview of therapeutic targets with reference to current views on pathophysiology. Some of the established drug targets are discussed as well as some more novel, putative targets and approaches.

2. OSTEOLYSIS RELATED TARGETS IN BONE METASTASIS

Bone metastases usually cause more skeletal damage than would be expected given the presence and volume of tumor cells alone. This is particularly true of the clinically lytic lesions seen in advanced breast cancer as opposed to the blastic bone metastases characteristic of advanced prostate cancer. It is now however becoming clear that even in bone metastases arising from prostate cancer there is a significant lytic component that is obscured by the blastic reaction (1). The major cause of osteolysis in bone metastasis is most likely to be the inappropriate or excessive activation of osteoclasts, the cells within bone whose physiological function is bone resorption. Osteoclasts are of haematopoietic stem cell origin and they develop via monocyte/macrophage lineage into mononuclear precursors and then into multinucleated quiescent cells. Healthy bone undergoes continuous turnover during life and the resorptive action of osteoclasts is balanced by deposition of new bone by osteoblasts. These cells originate from mesenchymal stem cells and are responsible for the production and secretion of the structural components of the bone matrix.

The central role of osteoclasts in bone metastasis has resulted in extensive investigation into the molecules and pathways with which they interact. These include parathyroid hormone related protein (PTHrP) and the system comprising of receptor activator of nuclear factor κβ (RANK) and its ligand (RANKL). The most effective therapy directed towards the

osteoclastic bone resorption to date has been that of the bisphosphonate class of drugs.

2.1 Parathyroid Hormone-Related Protein

Much of the investigation into the factors causing the osteoclast activation found in bone metastasis suggests that PTHrP is the principle mediator of this process (2). PTHrP was initially discovered during the search for a peptide sharing amino-terminal homology with parathyroid hormone from tumors associated with hypercalcaemia (3). PTHrP plays a central role in the hypercalcaemia that can occur in patients with malignant disease, particularly breast, ovarian, pancreatic lung and myeloma. It acts on the parathyroid hormone receptor to cause increased renal absorption and bone release of calcium (4).

Circulating PTHrP is difficult to detect in healthy subjects but is present in hypercalcaemic patients with breast cancer. Compelling evidence suggesting that PTHrP plays a key role in the development of osteolytic bone metastases comes from studies of tumor samples. Immunohistochemistry of 102 consecutive breast tumors revealed positive staining for PTHrP in 60% of cases (5). When a similar analysis was carried out on breast cancer metastases in bone, 92% of the samples showed PTHrP whilst only 17% of soft tissue metastases stained positively (6). Given the increased number of cells producing PTHrP in bone metastases it has been postulated that tumor cells expressing PTHrP enjoy a survival advantage in bone or that tumor cells that survive in bone are induced to produce PTHrP (7). In addition to this, positive staining for PTHrP in primary tumors has been shown to predict positively for bone metastases in patients who were followed up for a minimum of five years (8).

In the light of such findings implicating PTHrP in the development of osteolysis in metastatic bone disease, this protein represents a logical candidate for therapeutic intervention. This is supported by work in a murine model of osteolytic bone metastases: nude mice injected with a human breast cancer cell line showed reduced development of osteolytic bone metastases and a decreased bone tumor burden when treated with neutralizing antibodies to PTHrP as compared to control animals (2).

Signalling pathways involving PTHrP have also been investigated as therapeutic targets to abrogate its function. The ras protein p21, which is present in around 70% of primary beast tumors (9), has been shown to increase PTHrP production in tumor cells. In a model of hypercalcaemia where rat fibroblasts were transfected with the ras oncogene to induce PTHrP, mice injected with the cells demonstrated significant decreases in PTHrP levels when treated with a ras farnesylation inhibitor (10). More

recently, the p38 MAP kinase pathway has been shown to be an important effector of PTHrP production. It is well established that TGFβ stimulates PTHrP production (11, 12) and that it can signal through both Smad (13) and mitogen-activated protein (MAP) pathways (14). Partial reduction of TGFβ-stimulated secretion of PTHrP was achieved by transfecting MDA-MB-231 human breast cancer cells with dominant negative constructs of Smad family members. A complete inhibition of TGFβ-stimulated production of PTHrP was observed when this strategy was used in combination with p38 MAP kinase inhibition (15).

Vitamin D analogues are potentially another way of decreasing PTHrP induced osteolysis. Vitamin D itself has been shown to exhibit antitumor activities in a number of tumor cell lines including breast and prostate (16-18). Its use is often precluded in these cases because of its potent calcaemic activity. Therefore a number of vitamin D analogues have been developed which retain the antitumor activity of the naturally occuring hormone with lower calcaemic activities. In addition to their antitumor activities, vitamin D and vitamin D analogues are able to decrease PTHrP production by tumor cells (19). In the case of the PC3 prostate cancer cell line, this is brought about by reversing EGF induced PTHrP upregulation (20).

A further therapeutic avenue involving PTHrP is antagonism of its receptor. Recent studies have shown that both primary breast and prostate cancers and their bone metastases frequently express PTHrP and its receptor (21, 22). Blockade of the receptor may be efficacious both for treatment and prevention of bone metastases.

2.2 The RANK-RANKL System and OPG

RANK and its ligand, RANKL, form a recently identified cytokine system that regulates the proliferation, differentiation, activation and apoptosis of osteoclasts (23). This system, together with osteoprotegerin (OPG), which acts as a decoy receptor for RANKL, is currently receiving widespread attention since it represents a particularly attractive target for the management of a wide range of bone diseases.

RANKL, which has also been called TRANCE, osteoclast differentiation inducing factor and OPG ligand, is a member of the tumor necrosis factor (TNF) gene family. It is expressed by osteoblasts and other stromal cells and is has been shown that it is essential for the induction of osteoclasts from haematopoeitic stem cells and through all stages of their lifecycle (24, 25). Following the discovery of RANKL, most of the osteotropic factors, including PTHrP, that induce osteoclast formation and subsequent bone resorption have been shown to do so by causing upregulation of RANKL expression on the surface of osteoblastic cells (26, 27). RANKL then binds

to the RANK receptor on osteoclast precursors to induce osteoclast formation.

The central role of RANKL in osteoclast function has been shown *in vivo* by administering exogenous RANKL to normal mice. These mice had increased numbers and activity of osteoclasts accompanied by rapid bone loss, osteoporosis and severe hypercalcaemia (25). Mice in which the RANKL gene was disrupted, however, developed accumulation of unresorbed bone (osteopetrosis) and were completely lacking in osteoclasts (28).

RANKL activity can be blocked by OPG, a recently identified member of the TNF receptor family (29). OPG acts as a decoy receptor for RANKL and also binds TNF-related apoptosis-inducing ligand (TRAIL), another member of the TNF ligand family (30). It is produced by a variety of tissues and like RANKL its expression and production is regulated by a number of osteotropic cytokines, growth factors and hormones that regulate bone resorption. These interactions are well demonstrated by a "convergence hypothesis" as proposed by Hofbauer et al. and diagrammatically reproduced in Figure 1 (23). OPG counters the biological effects of RANKL: it inhibits the proliferation, differentiation and fusion of osteoclast precursor cells, inhibits the activation of mature osteoclasts and promotes their apoptosis (31).

The discovery and continuing characterization of the RANK-RANKL system along with OPG have identified these factors as key players in the final common pathway of the osteolysis that is so characteristic of metastatic bone disease. They hold much promise as targets for therapeutic intervention both for the prevention and treatment of bone metastases and preclinical studies have been encouraging. The initial *in vivo* studies of OPG demonstrated its ability to prevent bone resorption: transgenic mice overexpressing OPG (29) and normal animals treated with recombinant OPG (29, 32) both developed osteopetrosis. When ooprectomized rats, which are often used as a model for osteoporosis, were treated with recombinant OPG, the bone loss which these animals would otherwise suffer was prevented.

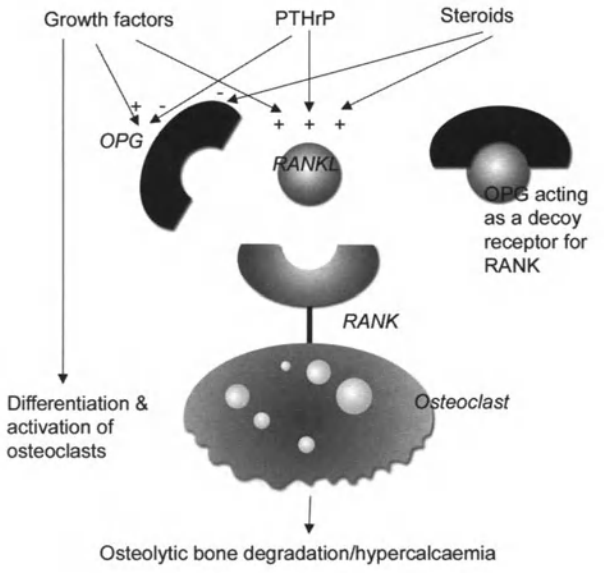

Figure 1. Diagrammatic representation of "convergence hypothesis" centred on RANKL and OPG as the final effector cytokines in malignant skeletal diseases. Many growth factors and cytokines converge at the level of RANKL and OPG to regulate osteoclast differentiation and function. Growth factors (such as interleukin-1β and tumor necrosis factor-α) stimulate RANKL and OPG production. PTHrP and other hormones such as glucocorticoids induce RANKL production but decrease OPG production. RANKL interaction with RANK leads to osteoclast differentiation but OPG can act as a decoy receptor to neutralise RANKL. Adapted from Hofbauer et al. (23).

These findings suggested that treatment directed at the RANK-RANKL system or utilizing the anti-resorptive properties of OPG would be useful in the setting of malignant disease and this has been the case in a number of more recent studies. The effect of OPG was assessed in a nude mouse model of osteolytic bone metastasis caused by injection of the MDA-MB-231 human breast cancer cell line. Histologically there was a 75% reduction in the skeletal tumor burden in the treated animals and eradication of tumor associated osteoclasts (33). Recent studies have reported the benefit of recombinant OPG treatment in murine models of multiple myeloma. Osteolytic bone lesions were prevented (34) and treated mice showed a significant increase in time to morbidity and increased survival (35). The preliminary results from a phase I clinical trial evaluating recombinant OPG therapy in patients with bone metastases secondary to breast cancer or multiple myeloma have been published recently (36). The activity of the

treatment was measured using a urinary marker of bone resorption, urinary N-telopeptide of collagen, and this showed a rapid and sustained decrease in treated patients. Interestingly, OPG treatment has also been evaluated in models of prostate cancer bone metastases on the basis that these lesions, conventionally thought of as osteoblastic, do in fact have an underlying osteolytic component. Prostate cancer cells were found to induce osteoclastogenesis from osteoclast precursors in the absence of a stromal component and to produce RANKL. When these cells were injected into the tibae of mice, OPG completely prevented the development of tumors, which were of a mixed osteolytic/osteoblastic phenotype in the control group (37). A similar effect was seen when human prostate cancer cells were injected into the intramedullary space of adult human bone implanted in SCID mice. In this model, said to yield osteoblastic tumors, OPG treatment resulted in inhibition of tumors and osteoclast numbers (38).

Another strategy aiming to inhibit RANKL activity has been the use of RANK-Fc, a recombinant form of RANK, to act as a decoy receptor for RANKL in a similar fashion to OPG. RANK-Fc is a fusion protein consisting of the extracellular domain of RANK and the constant region of human IgG_1. RANK-Fc has been shown to decrease bone destruction in mouse models of multiple myeloma (39). An advantage of RANK-Fc over OPG in this setting is that it does not bind TRAIL. This molecule has been shown to induce apoptosis in myeloma cells and is being investigated as a therapeutic agent itself (40). RANK-Fc has also been used in a model of humoral hypercalcaemia of malignancy in nude mice induced by PTHrP producing xenografts of human lung cancer. In this model, RANK-Fc prevented hypercalcaemia and the PTHrP associated osteolysis (41).

2.3 Bisphosphonates

Bisphosphonates, the focus of another chapter within this volume, represent the most successful class of drug directed towards the osteolytic process of bone metastasis. Amongst the targets and approaches considered in this review, only bisphosphonates are in clinical use. They are indicated for the prevention of skeletal related events in breast cancer and myeloma and for the treatment of bone pain in breast and prostate cancer (42). Recently, the most powerful of the bisphosphonates, zoledronic acid, has also been shown to be effective in preventing skeletal related events in prostate cancer.

Bisphosphonates are analogues of endogenous pyrophosphates in which the oxygen bridge is replaced with a carbon atom. A variable R′ chain determines the potency and the precise mechanism of action. The drug binds avidly to mineralized bone surfaces where it reaches very high

concentrations. Once released from the bone surface they are taken up by osteoclasts where they disrupt the biochemical processes involved in bone resorption and cause apoptosis of osteoclasts. There is evidence that bisphosphonates may also exhibit a broad range of anti-tumor activities including apoptosis of tumor cells, inhibition of tumor angiogenesis, tumor cell invasion and migration along with inhibition of proteolytic enzymes (43).

3. PROTEINASE RELATED TARGETS IN BONE METASTASIS

Amongst the critical steps in the pathogenesis of bone metastases is clearly destruction of the bone matrix in order to allow tumor expansion into bone. The activity of proteinase enzymes is necessary for invasion of tumors into soft tissue but is particularly important for the development of metastases in bone since this organ provides an abundant extracellular matrix that is particularly resistant to degradation. The matrix metalloproteinase (MMP) family of enzymes is that which has been studied in most depth in this regard and is discussed further below.

The urokinase plasminogen (uPA) activation system is the other major protease enzyme system responsible for degradation of the extracellular matrix (ECM). uPA together with its receptor uPAR classically promotes proteolysis by the activation of plasminogen to plasmin. A variety of other functions in the processes of invasion and metastasis have been ascribed to uPA/uPAR. These have been reviewed recently (44) and include regulation of both cell/ECM interactions via modulation of integrin function and cell migration by signal transduction pathways and direct chemotactic activity. There is good evidence that uPA/uPAR activity is associated with bone metastasis in breast and prostate cancers (45, 46) and intervention directed at this enzyme system holds promise at a number of levels.

Another example of a protease enzyme that may be inhibited to good effect and that may be even more specific to bone metastasis is cathepsin K. This serine protease capable of degrading bone matrix is primarily expressed by osteoclasts although its expression has also been seen in primary breast carcinomas (47). The use of antisense oligonucleotides to cathepsin K has been shown to inhibit osteoclast differentiation and bone resorption in an *in vitro* setting suggesting that it may be an effective therapeutic target for bone metastasis (48).

3.1 MMPs as Targets for Intervention in Bone Metastasis

The MMP family currently comprises 24 members which collectively are able to degrade all the components of the basement membrane and ECM. A wide range of extracellular proteins, including other proteinases, proteinase inhibitors, chemotactic molecules cell surface receptors and adhesion molecules serve as the substrates of MMPs (49). MMPs are intimately involved in bone remodelling: they promote osteoclast recruitment to sites of remodelling and are amongst the enzymes that degrade the mineralized bone matrix (50). The stromal compartment is the main source of MMP expression and production which is elevated in response to tissue damage or the presence of tumor cells. MMP expression has also been found in many tumors and has been shown to be present in clinical samples both in primary tumors and bone metastases (51). Potentially, therefore, therapy can be directed against MMP activity both in tumor and stromal cells.

The well established relationship between tumor progression and MMP overexpression has lead to the development of a number of MMP inhibitors. These have been evaluated as anti-cancer agents in clinical trials for various malignancies, although not specifically for bone metastases. The results of these trials have been disappointing with two being terminated due to poorer survival in the treatment arms compared to placebo. This subject has been powerfully analysed in a recent review which makes salient points with regard to clinical testing of molecularly targeted cytostatic agents (52). There are some important factors relating to MMPs and their inhibitors which should be appreciated when interpreting the results of clinical trials in which they did not demonstrate that investigators had hoped for. When the initial MMP inhibition trials were designed only three MMPs had been identified and now, in addition to the other family members, two further related families of MMPs have been identified – the ADAMs (a disintegrin and metalloproteinase) and the ADAMTSs (ADAMs with thrombospondin motifs) (53-55). Cross-inhibition of such a wide variety of targets had not been anticipated. Furthermore, the wide variety of biological effects of MMPs is only recently coming to light. Apart from matrix degradation, MMPs can mediate cell death, proliferation, differentiation, angiogenesis and malignant conversion. It may well be that activity of MMPs against some of the non-matrix substrates such as chemokines, growth factors, and adhesion molecules results in the rapid cellular responses that have been observed *in vitro* (49).

With the current and advancing understanding of MMP function, regulation and specificity along with the advances in the understanding of the pathophysiology of bone metastases, successful targeting of MMP

activity to treat or prevent bone metastasis is more likely. Recent *in vivo* studies support this view. It is known that prostate cancer cells produce MMPs when introduced into the bone environment (56). Using an *in vivo* model of bone degradation in which human prostate cancer cells are injected into human foetal bone fragments implanted in SCID mice, investigators have shown that the broad spectrum MMP inhibitor, batimastat, can prevent bone loss and reduce tumor cell proliferation (57). Pre-treatment with the anti-angiogenesis agent, Neovastat, which is also active against MMP-2, MMP-9 and MMP-12, (58) was shown to decrease bone tumor volume in nude mice subjected to intracardiac injection of MDA-MB-231 human breast cancer cells. These findings have been confirmed by another study using the same animal model and two different MMP inhibitors which were also shown to prolong survival (59).

3.2 Tetracyclines and MMP Inhibition in Bone Metastasis

Our laboratory has a particular interest in investigating the use of the tetracycline family of antibiotics in the treatment and prevention of bone metastases. Enthusiasm for this application for tetracyclines arose from studies in periodontal disease in which they were found to inhibit connective tissue via non-microbial mechanisms (60). Tetracylines are thought to inhibit MMPs by chelating zinc ions which are required for MMP activity (61). They also inhibit the activation of MMP proenzymes, the latent precursors of MMPs, prior to their activation by endogenous proteases (62). Chemically modified tetracyclines have been developed which retain their MMP inhibiting activity without maintaining their antibiotic activity. These have been shown to decrease bone loss in animal models of osteoporosis (63). The finding that tetracyclines induce apoptosis in osteoclasts lends further support to their use in the context of preventing bone loss in metastatic disease (64). *In vitro* studies of tetracycline have also shown that they are cytotoxic to both breast and prostate cancer cell lines (65-67). Recent investigation also suggests that tetracyclines can also inhibit angiogenesis (68). In addition to the anti-tumor and MMP activities exhibited by tetracyclines, they are particularly attractive as a therapy for bone metastasis on account of their osteotropism: they are quickly cleared from the circulation but accumulate in the skeleton where their activity is likely to be most beneficial (65).

Preclinical *in vivo* studies using tetracyclines in a bone metastasis setting have been encouraging. In a mouse model of bone metastasis, animals treated with the tetracycline derivative, doxycycline, showed a 63% decrease in bone tumor burden compared to untreated animals (69). Bone resorption

parameters and numbers of osteoclasts were lower in the treated group but bone formation was increased. This suggested that tetracyclines may also be beneficial in alleviating the uncoupling of rates of bone resorption and formation that are typically seen in osteolytic bone metastases. Phase II clinical trials evaluating doxycycline treatment versus placebo in breast and prostate cancer patients at risk of bone metastases are accruing patients at the authors' institution.

4. OTHER GROWTH FACTORS, PEPTIDES, CYTOKINES AND STROMALLY DERIVED FACTORS AS TARGETS FOR THERAPEUTIC INTERVENTION IN BONE METASTASIS

The list of growth factors, peptides and cytokines that are recognised to play a role in the development of bone metastases is continually expanding. The interaction between tumor cells and bone stromal factors, long recognised to be crucial for the development of metastases, is also becoming more clearly defined. Therapeutic targeting of stromal tissue is the long term goal of many of the studies of bone metastasis currently underway. Clearly not every molecule discovered to be involved in bone metastasis will hold therapeutic potential but refinement of our understanding of the mechanisms involved holds the key to effective therapeutic and preventative strategies in the future. Some of the more promising targets and mechanisms that have recently been demonstrated are now reviewed.

4.1 Endothelin-1

Endothelin-1 (ET-1) is one of a family of three 21 amino acid peptides initially identified as potent vasoconstrictors. Together with the endothelin A (ET_A) and endothelin B (ET_B) receptors, they form the endothelin axis which is gaining recognition for a wide variety of functions in the growth and progression of cancers (70). The activity of ET-1 is mediated largely through the ET_A receptor. It has varied effects on bone cells and is of particular interest in the pathogenesis of osteoblastic metastases. Osteoblast cells express both ET_A and ET_B receptors (71 ,72) and along with osteoclasts and vascular endothelial cells have been shown to stain positively for ET-1 itself (73). A number of experimental findings suggest that the activities are stimulatory to bone formation: it has mitogenic effects on osteoblasts (71, 72), inhibits bone resorption, (74) induces collagen synthesis (75) and alkaline phosphatase production (76). These results are controversial since in

a murine osteoblast precursor cell ET-1 has been shown to inhibit differentiation, reduce alkaline phosphatase activity and decrease *in vitro* mineralization (77, 78). These contradictory results may reflect differences in cell lines and ET receptor expression.

In vivo data with breast and prostate cancer cell models support the thesis that ET-1 promotes osteoblastic lesions. Investigators have identified a breast cancer cell line, ZR-75-1, that causes osteoblastic lesions in a mouse model of bone metastases. In contrast to MBA-MB-231 cells which cause osteolytic lesions, analysis of conditioned media from ZR-75-1, cells, was found to contain high levels of ET-1 (79). The conditioned media itself induced new bone formation in mouse calvarial organ cultures as did ET-1. Subsequent *in vivo* experiments have shown that ET_A receptor blockade reduced osteoblastic metastases caused by ZR-75-1 cells but the osteolysis caused by MBA-MB-231 cells was not affected (80). The ET_A receptor antagonist atrasentan (ABT-627) is currently being evaluated in a phase II clinical trial of prostate cancer patients with hormone refractory disease. Initial reports show that markers of bone deposition and resorption are stabilised in patients receiving atrasentan compared to placebo and that these findings were supported by those of clinical bone scan studies (81).

4.2 Stromally Derived Chemokines

The precise mechanisms by which some tumor cells preferentially metastasise to bone remain enigmatic. Three theories are commonly proposed to explain this phenomenon (82). One is that tumor cells leave the circulation in an even distribution across all organs but that they proliferate only in the organs which provide them with the appropriate growth factors. Another is that endothelial cells lining the vessels of the target organs express adhesion molecules that effectively trap the tumor cells. The third theory is one of chemoattraction: tumor cells utilise the chemokine pathway in order to home in on a particular organ such as the bone. Chemokines are similar in structure and function to growth factors. They are produced locally in tissues and bind to G-protein coupled receptors on leukocytes whose migration they are largely responsible for controlling (83). Stimulation of these receptors by chemokines induces the upregulation and activation of integrins enabling leukocytes to adhere to the vascular endothelial cells before migrating into tissues. The similarity in the behaviour of metastasising tumor cells led investigators to test the hypothesis that breast cancer cells express receptors that are activated by the same chemokines (84). They found that the chemokine receptors CXCR4 and CCR7 were highly expressed in breast cancer cells, malignant breast tumors and metastases. The chemokine ligands of these receptors, CXCL12/SDF-1 and

CCL21/6Ckine, were found to be preferentially expressed by tissues to which breast cancer commonly metastasizes: lymph nodes, lung, liver and bone marrow. Markedly lower expression was observed in skin, brain, kidney and skeletal muscle. They went on to show that in a mouse model of breast cancer metastasis, treatment with neutralizing antibodies to CXCR4 impaired the development of lung metastases.

This work has been supported by *in vitro* studies showing that prostate cancer cell lines express high levels of CXCR4 and that osteoblastic cell lines express high levels of SDF-1 (85). In order to assess whether chemokine activity might be involved in bone metastasis in prostate cancer, cells were treated with SDF-1 and found to adhere and migrate preferentially towards bone endothelial cells. *In vitro* invasion of prostate cancer cells was stimulated by SDF-1 but inhibited by CXCR4 antibodies. The expression of CXCR4 has also now been verified in clinical samples of prostate cancer by tissue microarray. CXCL12 mRNA was also detected at higher levels in metastatic than normal tissue (86). The involvement of chemokines in tumor biology is a rapidly advancing field of study (87) and the data reviewed here implies that they are critical regulators of metastasis to bone. Small molecule antagonists of chemokine receptors, under development for a variety of applications (88, 89), represent an attractive means of preventing or treating metastatic bone disease.

4.3 Macrophage Inflammatory Protein –1α

Macrophage inflammatory protein-1α (MIP-1α) is a potent stimulator of osteoclast activity and has recently been identified as mediator of osteolysis in myeloma. Recent data from *in vivo* experiments (90) have confirmed *in vitro* findings that MIP-1α stimulates osteoclast differentiation and bone resorption (91, 92). It is expressed in 70% of patient derived myeloma cells and at much higher concentrations in the supernatants of bone marrow from myeloma patients than those with other haematological malignancies or controls (93). Implantation of a myeloma derived cell line, ARH-77, into SCID mice, causes the animals to develop the clinical features of myeloma including osteolytic lesions and bone destruction. ARH-77 cells expressing an antisense construct to MIP-1α resulted in decreased tumor growth within bone marrow (93). In another mouse model of myeloma, utilising 5TGM1 cells, systemic treatment of animals with an antibody to MIP-1α led to decreased disease progression with significant decreases in bone tumor volume (90). The 5TGM1 cells used express the chemokine receptors CCR1 and CCR5, for which MIP-1α is a ligand (94). These and other chemokine receptors seem to be implicated in the migration and homing mechanisms of myeloma cells to bone. This again offers the opportunity of targeting the

chemokine receptor pathway in the management of metastatic bone disease (95-98).

4.4 Platelet Derived Growth Factor

Platelet derived growth factor (PDGF) is a peptide whose originally described target cells were fibroblasts and smooth muscle cells. It is a pro-angiogenic molecule that signals through a cell surface tyrosine kinase receptor (PDGF-R) and also mediates various cellular functions including growth, proliferation and differentiation (99). Coexpression of PDGF and PDGF-R occurs in a number of malignancies and is suggestive of autocrine stimulation of tumor growth by this growth factor system. A recent study of a mouse model of breast cancer metastasis to bone has shown that breast cancer cells overexpressing the HER-2-NEU oncogene produced large amounts of PDGF (100). A causative role for PDGF was suggested in the development of osteosclerotic metastases in this model. Further evidence of PDGF signalling in bone metastasis has been shown in a study in which ST571 (imatinib mesylate, Gleevec) was used to antagonise PDGF-R (101). This inhibitor of the Abl and BCR-Abl tyrosine kinases has demonstrated a high degree of efficacy in the treatment of chronic myeloid leukaemia (102) and is also a potent inhibitor of PDGF-R (103). Human prostate cancer cells were injected to the tibias of nude mice and the animals were divided into different treatment groups, one of which was treated with oral ST571. Tibias from the treated mice had lower tumor incidence, smaller tumors and less bone lysis than untreated controls. There was less phophosphorylated PDGF-R on the tumor cells of treated mice than controls indicating that the beneficial effect of ST571 was mediated through this tyrosine kinase receptor. This example of targeting a growth factor pathway to abrogate the development of bone metastases is likely to be joined by other similar approaches as the role of growth factors and their receptors in bone metastasis gains further definition.

4.5 Osteopontin

Osteopontin is a secreted glycoprotein and cytokine that is produced by osteoclasts, macrophages, T-cells and the kidney (104). High levels of osteopontin expression are characteristic of metastatic cancer (105, 106) and plasma levels have been correlated with the presence of bone metastases in prostate cancer (107). When melanoma cells were inoculated into osteopontin deficient mice, there was a significant decrease in bone metastasis compared to that seen in wild type control animals (108). Various mechanisms for this effect were proposed in that study but one involving the

binding of osteopontin to integrins is attractive. Osteopontin is sensitive to a number of proteases and following cleavage it divides into two functional domains. The N-terminal fragment ligates members of the integrin family and in particular integrin $\alpha v\beta 3$. This integrin is consistently expressed by breast cancer metastases and promotes osteolysis and bone metastasis (109, 110). Osteopontin and its interacting partners are promising factors at which to direct anti-metastatic therapy and a number of potential strategies have recently been reviewed (104).

4.6 Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs) belong to the TGF-β family of growth factors and comprise over 30 members originally characterised by their ability to induce ectopic bone formation when implanted under the skin of rodents (111). Much of the investigation concerning BMPs is directed towards applying their ability to cause osteogenic differentiation to bone healing and skeletal regeneration in orthopaedic surgery (112). This osteogenic activity has also sparked interest in the possibility that BMPs could be involved in the genesis of osteoblastic metastases. Data from studies of prostate cancer demonstrate the expression of BMPs to be associated with bone metastases (113, 114). Further mechanistic studies may reveal these proteins to be candidates for therapeutic intervention against bone metastasis.

5. CONCLUSIONS

As the mechanisms underlying the pathophysiology of bone metastases become more clearly defined, so the list of potential therapeutic targets increases. The reasons for cancer cells to home to bone have been elusive but evidence points towards chemokines as important factors. Treatment directed at these offers the hope that bone metastases may be prevented or significantly delayed in high risk individuals. Growth factor function, production and their targets similarly hold promise as molecular sites that may be used to decrease tumor growth. In addition unravelling of the secrets of normal bone physiology continues to provide clues to the response of bone to tumor and its action upon the tumor. This effectively doubles the targets available for intervention. Bisphosphonates have been shown to be an efficacious highly targeted therapy. Understanding the principles underlying osteolysis, particularly the RANK/RANKL system, OPG and PTHrP activity, mean that bisphosphonate therapy may be combined with other approaches in order to further increase efficacy. This is complemented by

appreciation that many metastases are of mixed osteolytic/osteoblastic morphology and identification of osteoblastic factors such as ET-1 potentially allows intervention directed at the uncoupling of bone formation that is so destructive in metastatic bone disease. The diagram in Figure 2 is a representation of some of the mechanisms, sites, current and proposed interventions for dealing with bone metastases. The data reviewed here encompasses identification of the mechanisms of bone metastasis and *in vivo* evaluation of approaches. Some of these approaches have already reached the stage of clinical trials and others are sure to do so in the near future. This should translate into a welcome and tangible clinical benefit.

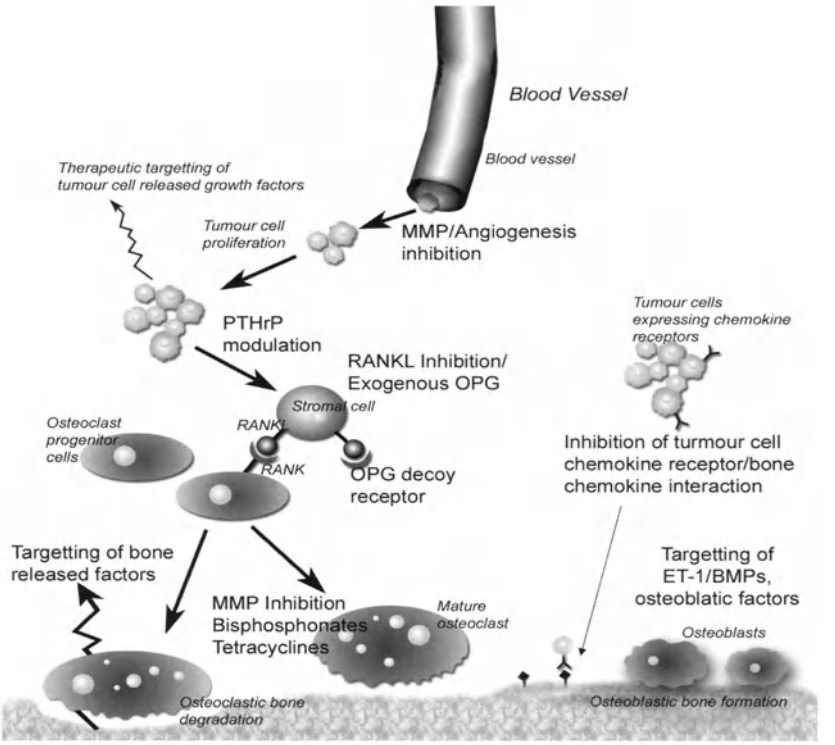

Figure 2. Processes involved in the development of bone metastasis and sites at which therapeutic intervention may be directed.

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Chapter 11

OPG, RANK AND RANKL IN BONE METASTASIS AND CANCER-ASSOCIATED OSTEOLYSIS

Paul J. Kostenuik
Research Scientist, Amgen Inc., Thousand Oaks, California, USA

1. INTRODUCTION

A triad of recently discovered proteins has dominant effects on most aspects of physiological and pathological bone resorption. The discovery of these proteins helped to resolve numerous questions regarding the exact molecular mechanisms for bone resorption. These proteins also neatly account for the long-recognized dependence of osteoclasts on the osteoblast/stromal cell population. This pathway was rapidly elucidated after the discovery of osteoprotegerin (OPG), an endogenous protein that negatively controls most aspects of osteoclast formation, activation and survival. The biological counterpart for OPG is RANKL (receptor activator of nuclear factor-κB ligand), a protein that promotes osteoclast formation, activation and survival. RANKL functions by binding to its receptor RANK on the surface of osteoclasts and their precursors. RANKL is the final mediator of osteoclast activation induced by most, if not all, proresorptive cytokines.

In the simplest terms, bone resorption is initiated by RANKL binding to RANK. Bone resorption is arrested when OPG binds and neutralizes RANKL, thereby preventing the latter from binding to RANK (Figure 1). An important take-home message is that bone resorption is governed by the relative abundance of OPG and RANKL (Figure 2). A relative excess of RANKL leads to bone resorption, while a relative excess of OPG arrests

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bone resorption. A variety of tumor-derived growth factors and cytokines can influence the expression of OPG and RANKL. The ultimate effect of tumor derived factors on bone resorption cannot be accurately predicted without accounting for changes in the relative abundance of both OPG and RANKL. Most if not all forms of pathologic bone resorption rely on the OPG/RANK/RANKL pathway. Conditions that involve accelerated bone resorption, ranging from rheumatoid arthritis to spaceflight, appear to cause bone loss via this axis. Cancer is among the more common conditions that involve pathological bone loss, and cancer cells have evolved several mechanisms to commandeer the OPG/RANK/RANKL pathway to activate osteoclasts. Some tumors appear to produce their own RANKL to directly activate osteoclasts, while others produce factors that induce RANKL expression by other cells such as osteoblasts and stromal cells. Other tumor types, such as multiple myeloma, may sequester OPG and thereby increase the relative abundance of RANKL.

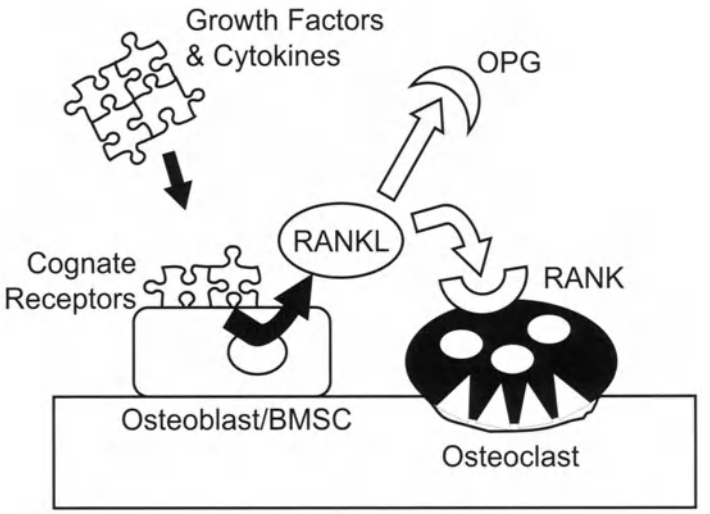

Figure 1. Basic biology of the OPG/RANK/RANKL axis. Various growth factors and cytokines bind to their cognate receptors on osteoblasts or bone marrow stromal cells (BMSCs). Signaling of these receptors may culminate in the production of RANKL. Osteoblasts/BMSCs also produce OPG, a soluble decoy receptor that can bind and neutralize RANKL. If RANKL is not bound by OPG, it is able to bind to RANK on osteoclasts and osteoclast precursors. RANKL binding to RANK causes osteoclast formation, activation and survival, resulting in increased bone resorption.

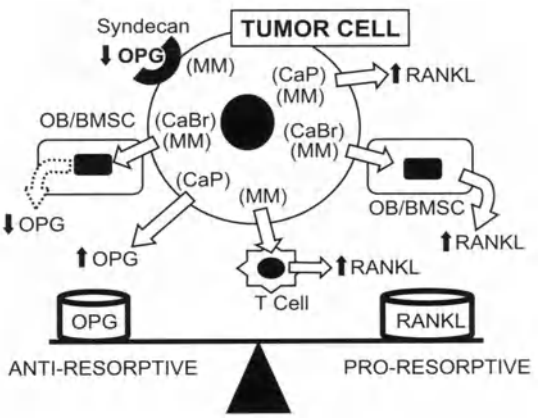

Figure 2. Bone resorption is governed by the relative abundance of OPG (anti-resorptive) and RANKL (pro-resorptive). Tumor cells utilize several mechanisms to upset the balance of OPG and RANKL to favor osteolysis. Clockwise from upper right: Prostate Cancer (CaP) and Multiple Myeloma (MM) cells may produce RANKL directly. Breast Cancer (CaBr) and MM cells may induce RANKL production from osteoblasts and bone marrow stromal cells (BMSC). MM cells may induce T cell production of RANKL. CaP cells can secrete OPG directly, which might serve to inhibit osteolysis. CaBr and MM cells may suppress the production of OPG by osteoblasts and BMSCs. MM cells may bind and degrade OPG via Syndecan-1.

While the OPG/RANK/RANKL axis may neatly account for the mechanisms of osteolysis, the mechanisms responsible for cancer cell osteotropism remain unclear. It was recognized over 100 years ago that breast cancer cells have a special prediliction for bone. Other neoplasms, including multiple myeloma, also have a propensity to spread and grow within the skeleton. The growth of these metastases is usually accompanied by focal osteoclast-mediated osteolysis that can lead to pain, hypercalcemia, immobility and death. The reasons why certain malignancies tend to thrive in bone remain unclear and are likely to be multifactorial. However, a recurring conclusion from animal studies of experimental bone metastasis is that bone resorption often favors skeletal tumor growth. The ability of many bone-metastasizing tumors to increase bone resorption may create a vicious cycle of bone destruction and tumor growth. This chapter will summarize an emerging body of literature that implicates RANKL as a major culprit in this pathological process.

The chapter will first discuss the basic biology of the OPG/RANK/RANKL pathway. Attention will then shift to describing the regulated expression of these proteins in cells and tissues that are associated with bone metastases. Studies that describe the localization and regulation of these proteins have been tremendously informative to our understanding of

osteolysis. Bone metastasizing tumors of different origins have adopted several mechanisms that can disrupt bone remodeling. For osteolytic tumors, such as myeloma and breast carcinoma, these mechanisms all converge on the OPG/RANK/RANKL pathway. A better understanding of these mechanisms may eventually provide the foundations for a better understanding of tumor osteotropism. The final chapter section describes the pharmacologic effects of RANKL antagonists in animal models of bone metastasis. OPG is the only known endogenous antagonist for RANKL, but other types of RANKL antagonists (anti-RANKL antibodies, soluble RANK-Fc) are also very effective at protecting bone. These observations further implicate RANKL as a major etiologic agent in osteolysis while providing additional tools for dissecting the pathophysiology of bone metastasis. It is hopeful that some of these antagonists will become effective therapeutic tools for the treatment of patients with bone metastasis.

2. BASIC BIOLOGY OF OPG/RANK/RANKL

Several comprehensive reviews have been published on the OPG/RANK/RANKL pathway (1-3). This chapter will attempt a brief description of the axis within the context of tumor-associated osteolsysis. OPG [also known as TR1 (4), FDCR-1 (5), OCIF (6)] is a member of the tumor necrosis factor (TNF) receptor superfamily. It was originally discovered as an expressed sequence tag from fetal rat intestine (7), and was independently identified as an osteoclast inhibitory factor from cultured human fibroblasts (6). OPG is unique among members of the TNF receptor family in that it is a secreted protein with no known signaling functions. OPG is a heparin-binding glycoprotein that can exist as a 55 kD monomer or a 110 kD homodimer (7). OPG acts as a decoy receptor by binding to its natural ligand RANKL (Figure 1). OPG is clearly important to normal bone physiology, as indicated by the phenotypes of mice that either over-express or fail to express OPG. The systemic overexpression of OPG in transgenic mice causes severe osteopetrosis associated with a near total lack of osteoclasts (7). In contrast, ablation of the OPG gene in knockout (KO) mice causes severe osteoporosis and increased osteoclast numbers (8). OPG KO mice are osteopenic within a week of birth and spontaneous skeletal fractures occur as early as two weeks post partum. The loss of even a single allele of the OPG gene results in progressive osteopenia (8).

RANKL (9) is a member of the TNF ligand family and is also known as OPGL (10), TRANCE11 and ODF (12). RANKL can be expressed as a type II transmembrane protein on the surface of several cell types, including osteoblasts (13), stromal cells and T-cells (9). This transmembrane form is

likely to account for the requirement for cell-to-cell contact in some culture models of osteoclastogenesis (14). A soluble form of RANKL has also been identified (15), which may account for osteoclastogenesis in systems where cell-to-cell contact is not required (16). RANKL KO mice have a phenotype that closely resembles that of the OPG transgenic mice: severe and progressive osteopetrosis with a near total lack of osteoclasts. Osteopetrosis is evident in RANKL KO mice within 2 days of birth (17).

RANK, like OPG, is a member of the TNF receptor superfamily. Unlike OPG, RANK is a transmembrane receptor that signals through nuclear factor-κB (NF-κB) and Jun N-terminal Kinase (JNK) (18, 19). Virtually all known cellular responses to RANKL are mediated by RANK. RANK was originally identified as a receptor on dendritic cell that promoted their survival 9, but gene ablation studies in mice revealed the dominant role of RANK in bone. RANK KO mice have severe osteopetrosis due to a nearly complete lack of osteoclasts (18, 20). Transgenic systemic overexpression of a truncated extracellular form of RANK (RANK-Fc) also causes severe osteopetrosis (7). This transgenic model phenocopies that of the OPG transgenic model, which is consistent with the mechanism of action for OPG and for RANK-Fc: to bind RANKL and prevent its interaction with RANK.

The consistent and predictable phenotypes from these OPG/RANK/RANKL transgenic and knockout studies leave little room for additional players in the ultimate control of bone resorption. It has been proposed that pro-resorptive cytokines all converge on this axis to regulate bone resorption6. For example, RANKL, parathyroid hormone related protein (PTHrP), $1,25(OH)_2D_3$, IL-1β and TNF-α each cause increased osteoclast numbers and elicit hypercalcemic responses in wild-type mice, but not in RANK KO mice (20). The hypercalcemic and osteoclastic response to each of these factors can also be blocked in normal mice by recombinant OPG (21). These observations suggest that RANK/RANKL interactions are the final mediators of bone resorption induced by a wide variety of pro-resorptive factors.

An advantage to this "convergence" theory is that it may narrow the search for new biological agents and chemical entities that can block cancer-associated bone resorption. In contrast to the variety of upstream factors that indirectly mediate bone resorption (22), there are only three therapeutic targets within the OPG/RANK/RANKL axis and each of these may be interdicted by various therapeutic modalities. OPG is the natural inhibitor of RANKL, but other molecules have been engineered to function in analogous manner. For example, soluble RANK constructs that lack signaling potential are effective and selective inhibitors of RANKL (19, 23, 24). Monoclonal antibodies that neutralize RANKL are also effective and highly selective inhibitors (25).

3. EXPRESSION AND REGULATION OF OPG/RANK/RANKL IN BONE METASTASIS

The OPG/RANK/RANKL proteins are expressed by many of the cells that are considered important to the establishment and progression of bone metastases. Evidence of their altered expression in bone metastasis suggests that they may be more than passive participants. This section summarizes data that confirms the important roles of OPG/RANK/RANKL in cancer-related osteolysis. Efforts are underway to explore the potential diagnostic and prognostic value of these proteins. The majority of studies have focused on carcinoma of the prostate and breast, as well as multiple myeloma. The OPG/RANK/RANKL pathway has also been implicated in the osteolysis associated with giant cell tumors. Emerging data suggest that each of these malignancies may utilize the OPG/RANK/RANKL pathway in different ways. A fundamental question for each tumor type is whether osteolysis is induced directly by the tumor cells or indirectly by directing the expression of RANKL by host cells in bone.

3.1 Multiple Myeloma

Multiple myeloma is a plasma cell malignancy that is typically confined to bone marrow, where it induces significant osteolysis. Of the neoplastic diseases that affect bone, multiple myeloma has generated the greatest amount of interest with regards to the OPG/RANK/RANKL pathway (Figure 2). Emerging evidence suggests that the OPG/RANK/RANKL pathway is influenced by myeloma cells at a number of levels, and each of these influences would predict an increase in osteoclast activity. It has been acknowledged for decades that myeloma cells produce an "osteoclast stimulating factor", or factors (26). Several myeloma-derived factors have been strongly implicated in promoting osteolysis (reviewed in 22, 27). Many of these factors, including IL-6 (28, 29), IL-1β (21,30), PTHrP (21) and TNF-α (21, 31, 32), are likely to induce bone resorption by stimulating the production of RANKL and/or by reducing the production of OPG in osteoblasts or stromal cells. Myeloma cells also induce IL-6 expression by osteoblasts (33), which could then induce RANKL expression by osteoblasts through an autocrine route.

It is perhaps not surprising that bone marrow biopsies have revealed elevated RANKL in association with myeloma cells (23, 34). Several mechanisms have been proposed to explain increased RANKL levels in multiple myeloma, most of which involve myeloma cell upregulation of RANKL by local host cells in bone. In co-culture experiments, myeloma cells upregulated the mRNA expression of RANKL by osteoblasts and

stromal cells (23, 35) and also in T cells (36) (Figure 2). Immunohistochemistry of human bone marrow samples also suggests that myeloma cells induce RANKL expression by osteoblasts and stromal cells (23, 34, 35). T cells may also be an important source of RANKL in myeloma patients. T lymphocytes isolated from the bone marrow of myeloma patient with severe osteolysis expressed RANKL mRNA, while those cells from healthy controls or patients without osteolysis did not express RANKL (36). Conditioned medium from cultured T cells was capable of inducing osteoclastogenesis from precursors that were co-cultured with human myeloma cell lines. This osteoclastogenic stimulus was inhibited by OPG 36, which is consistent with the ability of OPG to neutralize RANKL regardless of its cellular origin.

Whether myeloma cells directly express RANKL remains controversial. Most immunohistochemistry studies failed to detect RANKL protein associated with myeloma cells, despite the fact that RANKL was detected in association with osteoblasts, stromal cells and T-cells from the same samples (23, 34, 35). Furthermore, ten human myeloma cell lines and primary myeloma cells isolated from 26 consecutive patients failed to express RANKL mRNA (35). Perhaps the most compelling data to identify the cellular source for RANKL comes from co-culture experiments, wherein several human myeloma cell lines were capable of inducing osteoclastogenesis from mixed cultures of marrow cells and stromal cells taken from normal mice (23). When the murine stromal cells were derived from RANKL knockout mice, the myeloma cell lines were no longer capable of inducing osteoclastogenesis. This observation suggests that normal host cells, rather than myeloma cells, are the critical source of RANKL. In contrast to these results, immunohistochemistry of cytospins and marrow smears from multiple myeloma patients revealed myeloma cell expression of RANKL (37). Flow cytometry of marrow from myeloma patients also revealed RANKL expression by plasma cells (38). There is good evidence that at least one murine myeloma cell line (5TMM) expresses RANKL mRNA and protein, which may account for the ability of this cell line to produce osteolytic lesions in syngeneic mice (39). It is clear that more analyses will be required from independent labs, using various detection methods, to reach any concensus on whether primary human myeloma cells consistently express RANKL.

Regardless of the exact underlying mechanism, the induction of RANKL in association with myeloma cells would by itself predict an increase in bone resorption that could account for myeloma-related osteolysis. Changes in OPG levels should have the same pathologic consequences as changes in RANKL levels, and myeloma is the only neoplasm described thus far that is associated with reduced levels of OPG. This phenomenon would tend to

exacerbate osteolysis, particularly when accompanied by the induction of RANKL. Patients with myeloma had reduced OPG levels in their serum (35, 40) and in bone marrow plasma (41). Myeloma patients with lytic disease also had lower serum OPG levels than those without lytic disease, and lower OPG levels were associated with poorer WHO performance status (42). Serum OPG levels did not however predict treatment response or survival in those patients. Several hypothetical mechanisms have been proposed to explain the decreased OPG levels observed in myeloma patients, and there is little reason to believe that these mechanisms are mutually exclusive. Osteoblasts and stromal cells are major contributors to the OPG pool, and it is possible that myeloma causes reduced OPG production by these cells. In support of this notion, OPG mRNA expression by osteoblasts or stromal cells was suppressed when co-cultured with human myeloma cells (Figure 2) (23, 35). In human bone marrow biopsies, the presence of myeloma was associated with reduced stromal cell and osteoblastic expression of OPG mRNA (23, 35). Serum OPG levels in myeloma patients correlated with a serum marker of bone formation (42), suggesting that a general suppression of osteoblast function in advanced myeloma could lead to reduced OPG production.

Another intriguing hypothesis for reduced OPG levels in myeloma patients is that myeloma cells themselves may bind and sequester OPG (Figure 2). Several myeloma cell lines, as well as primary myeloma cells, have been shown to bind human OPG in tissue culture studies (41, 43). OPG binding to myeloma cell membranes may be mediated by syndecan-1, a transmembrane proteoglycan with heparan sulfate side chains that is expressed on normal and malignant plasma cells (Figure 2) (41). Native OPG has a highly conserved heparin binding domain (7) that is presumably the target for syndecan binding, as OPG binding to syndecan-1 was greatly reduced in the presence of soluble heparin (41). OPG can be rapidly internalized and degraded after binding to cultured myeloma cells, a phenomenon that may contribute to the reduced OPG levels in myeloma patients (41). It remains to be proven whether myeloma cells can directly bind and neutralize OPG in vivo, but it is interesting to note that plasma cells in the bone marrow of myeloma patients stain positive for OPG protein (41). This staining did not appear to result from endogenous OPG production, as myeloma cell lines did not express OPG mRNA or protein (35, 41). It is worth noting that the heparin binding domain of OPG is not necessary for its binding to RANKL (7), and OPG lacking the heparin binding domain did not bind to myeloma cells (43). Therapeutic forms of OPG lack this heparin binding domain, and it is therefore probable that these drugs would avoid the myeloma cell binding and degradation fate while maintaining their antiresorptive potential (44).

3.2 Breast Cancer

Bone metastasis from breast cancer is typically associated with focal osteolysis, and a variety of breast cancer-derived factors have been implicated as osteoclast activating factors (reviewed in 22). Breast cancer cells may be capable of releasing soluble factors that directly activate osteoclasts (45), but it is unlikely that many breast tumors directly produce RANKL. In one study, twelve of twelve primary breast tumors failed to express RANKL mRNA (46), and another study failed to demonstrate RANKL expression by immunohistochemistry (47). Human breast cancer cell lines, including MDA-231, MCF-7 and T47D, fail to express RANKL mRNA (46, 48, 49). The majority of breast cancer-derived factors appear to stimulate osteoclasts indirectly. Before the discovery of OPG/RANKL, it was appreciated that many breast cancer-derived factors act on cells of the osteoblast lineage to induce bone resorption (50). In fact, the presence of osteoblasts or stromal cells is typically required in order for breast cancer cells to induce osteoclastogenesis (46, 51, 52). This requirement may be explained by data from co-culture studies, wherein the presence of breast cancer cells caused osteoblasts or stromal cells to increase their production of RANKL while reducing their production of OPG (Figure 2) (46, 51). This reciprocal control of OPG and RANKL might serve to amplify the pro-resorptive signal by increasing the relative abundance of RANKL. The addition of exogenous OPG to these co-culture systems blocked the ability of breast cancer cells to induce the osteoblast-mediated stimulation of osteoclasts (52).

Breast cancer cells probably secrete a number of pro-resorptive factors that act via osteoblasts and stromal cells, and the best characterized of these factors is PTHrP. PTHrP is frequently produced by human breast cancer cells (53), and tumor-derived PTHrP is a common catalyst for osteolysis and hypercalcemia (54, 55). PTHrP alone is able to mimic many of the activities of breast cancer cells in co-culture models of osteoclastogenesis. For example, PTHrP requires osteoblasts or stromal cells to exert its osteoclastogenic influence (56). The binding of PTHrP to PTH/PTHrP receptors on these cells leads to the stimulation of RANKL (46, 49) and the suppression of OPG (46). PTHrP overexpression by breast cancer cells is sufficient to induce RANKL, suppress OPG, and stimulate osteoclastogenesis in co-cultures that include osteoblasts (46).

Breast cancer-derived PTHrP also appears to use similar mechanisms in vivo to promote osteolysis. In a nude mouse calvarial injection model, the expression of PTHrP by human MCF-7 breast cancer cells was associated with increased RANKL expression by osteoblasts and the induction of bone resorption (49). MCF-7 cells expressing PTHrP were also better able to

survive and migrate along the calvarial surface compared to MCF-7 cells that did not produce PTHrP. While PTHrP has a well-defined role in mediating breast cancer-associated osteolysis, the role of PTHrP in promoting bone metastasis and osteotropism is less clear. Elegant studies have supported a causal role for PTHrP in animal models of bone metastasis (57), while data from other studies using similar models have argued against this role (58). Clinical data has suggested that PTHrP production by breast cancer cells is associated with improved survival and reduced bone metastasis (59). While there is a lack of concensus regarding the role for PTHrP in the development of bone metastasis, there is little debate that PTHrP promotes bone resorption through mechanisms that are dependent on OPG/RANK/RANKL (20).

Despite our improved understanding of osteolysis in breast cancer, the mechanisms responsible for breast cancer osteotropism are still poorly understood. Recent observations from RANK knockout mice have provided the basis for a new hypothesis for osteotropism that warrants vigorous testing and challenges. Breast cancer cells frequently express RANK mRNA 46, but the function or consequences of this expression is unknown. Potential clues for RANK function on breast cancer cells are provided by examining the role of RANK in normal mammary epithelial cells, from which most mammary carcinomas arise. The expression of RANK protein is highly restricted (9, 20), so it was somewhat surprising to learn that murine mammary epithelial cells also expressed RANK (60). A functional role for RANK on these cells was strongly supported by observations that mammary gland development during pregnancy was completely arrested in mice lacking RANKL. RANK knockout mice had similar mammary gland defects and were also unable to lactate. Supplementation of RANKL knockout mice with recombinant RANKL rescued the mammary defect, but RANKL supplementation did not rescue the defect in RANK knockout mice. These data indicate that RANK signaling was necessary and sufficient for mammary development during pregnancy (60). RANK was constitutively expressed by mammary epithelial cells, while RANKL was not constitutively expressed but was strongly induced during pregnancy. This induction was associated with alveolar epithelial cell proliferation, avoidance of apoptosis, and ultimately the formation of lobulo-alveolar structures that support lactaction (60). The injection of progesterone, prolactin or PTHrP also induced RANKL expression in mouse mammary tissue, suggesting that these factors may account for the pregnancy-related induction of RANKL.

It is unclear whether the human mammary gland uses similar pathways to regulate lactation during pregnancy. A more provocative question is whether mammary epithelial cells retain functional RANK expression after neoplastic transformation. Early data support this possibility, as RANK

mRNA expression was demonstrated in all twelve primary breast cancers analyzed (46). Human breast cancer cell lines also express RANK mRNA (46) (and our unpublished data). The expression of functional RANK protein has been reported in human Hodgkin lymphoma cell lines (61) but evidence for functional RANK on breast cancer cells is still lacking. Recent abstract data indicates that RANKL induces responses in breast cancer cells. These responses include NF-κB activation, increased mRNA expression of TGF-β and PTHrP, as well as invasion through matrigel (62). These observations are consistent with the presence of functional RANK on breast cancer cells, although this conclusion is based strictly on the apparent lack of alternative signaling pathways for RANKL (9, 20). If breast cancer cells express functional RANK, then it is conceivable that they could proliferate and invade in response to RANKL in the same manner as would mammary epithelial cells during pregnancy. The ability of bone-derived RANKL to promote breast cancer cell proliferation and survival would provide an elegant hypothetical mechanism for breast cancer cell osteotropism. An extension of this hypothesis is the potential for anti-RANKL therapies to have direct anti-tumor effects on breast cancer cells superimposed on their well-established and potent antiresorptive effects. Clearly there are substantial gaps in the body of data that would support this hypothesis of osteotropism, but unfortunately there is also a paucity of internally consistent and testable alternative hypotheses.

3.3 Prostate Cancer

Bone metastases from prostate cancer are typically blastic in nature, so it is not immediately intuitive that OPG/RANK/RANKL would have a significant role in this process. However, most bone metastases from prostate cancer also include an osteolytic component (63-66) and prostate cancer patients with bone metastases frequently have elevated serum markers of bone resorption (67). The stimulus for this osteolytic component is not well understood, and it may merely represent a normal coupling phenomenon that follows prostate cancer stimulation of osteoblasts (68). Recent data suggest that prostate cancer cells may also play a more direct role in stimulating osteoclasts. A bone-metastasizing human prostate cancer cell line (C4-2B) secretes a soluble form of RANKL that directly promotes osteoclastogenesis (Figure 2), an effect which is blocked by OPG (16). Primary human prostate cancer cells have also been shown to express RANKL mRNA, and this expression was apparently upregulated in bone metastases (69). The significance of these observations and their influence on bone resorption are difficult to determine, as OPG mRNA was also elevated in prostate bone metastases (69).

The expression of OPG by prostate cancer cells may explain the recent observations that patients with advanced prostate cancer have elevated serum levels of OPG (70). Prostate cancer patients with bone metastasis were also reported to have increased serum OPG levels compared to prostate cancer patients without bone metastasis (71). In this patient panel, serum OPG levels had some diagnostic value for identifying and discriminating patients with bone metastasis. The cellular source for this increase in serum OPG is an important unanswered question, but the obvious candidates are osteoblasts or prostate cancer cells. Prostate cancer cells typically stimulate osteoblasts, which are a rich source of OPG (13). Several human prostate cancer cell lines have been shown to secrete OPG (Figure 2), and immortalized human prostate cells also secrete OPG (72).

3.4 Giant Cell Tumors and Other Neoplasms

Giant cell tumors are osteolytic tumors that originate in bone. These tumors are not commonly metastatic, but they represent useful and well-defined model system for studying tumor cell stimulation of osteoclasts. Giant cell tumors are characterized histologically by neoplastic mesenchymal stromal cells surrounded by mononuclear precursors and large multinucleated osteoclast-like cells (73). These multinucleated "giant cells" cause significant osteolysis, and the molecular mechanisms leading to their formation and activity have been largely exposed with the discovery of OPG/RANK/RANKL. Immunohistochemistry and in situ analysis reveals RANKL expression in giant cell tumors (47), and this expression is confined to the neoplastic stromal cell component (74-77). The giant cells express RANK but not RANKL 74-77. The restricted expression of RANK and RANKL in giant cell tumors is reminiscent of the normal expression pattern found in osteoclasts and bone marrow stromal cells. Neoplastic transformation of normal stromal cells could initiate their unrestrained recruitment and stimulation of osteoclasts via RANKL. RANKL mRNA expression was indeed higher in giant cell tumors compared to normal bone (76). OPG was also expressed in giant cell tumors, but there was no apparent increase in OPG mRNA levels compared to those found in normal bone (76). These qualitative differences in mRNA levels would predict a relative excess of RANKL protein in giant cell tumors, which may account for their osteolytic nature. The fact that OPG blocks the resorption of cultured bone slices by giant cell tumors further supports this notion and provides some rationale for the use of RANKL antagonists in the treatment of giant cell tumors (74).

Neuroblastoma is a childhood neoplasm that can result in osteolytic bone metastases. Recently a nude mouse model was developed to characterize the

spread of human neuoroblastoma cells to bone. Subcutaneous injection of the human neuroblastoma cell line NB-19 resulted in the development of osteolytic lesions in bone (78). These NB-19 cells also induced osteoclastogenesis when co-cultured with bone marrow containing stromal cells and osteoclast precursors, and this response was blocked by the addition of OPG (78). In these co-cultures, the presence of NB-19 cells was associated with the induction of RANKL mRNA, while OPG mRNA levels were unaffected by the presence of NB-19 cells. The cellular origin of RANKL in this system was not identified, but it is likely that the NB-19 cells were either directly producing RANKL or they were inducing RANKL expression by stromal cells.

Adult T-cell leukemia (ATL) is a non-metastatic neoplasm with a high incidence of hypercalcemia. The hypercalcemia is associated with increased osteoclasts and pathological bone resorption. ATL cells from patients with hypercalcemia were recently shown to express high levels of RANKL mRNA (79). These ATL cells induced osteoclastogenesis from human bone marrow precursors in the presence of M-CSF, and OPG blocked this osteoclastogenesis. ATL cells isolated from patients without hypercalcemia expressed lower levels of RANKL and did not induce osteoclastogenesis. These observations led the authors to hypothesize that RANKL expression is the most important factor in the development of hypercalcemia in ATL patients (79). Other hypercalcemia-inducing tumors may also have the capacity to directly secrete RANKL. For example, a human squamous carcinoma cell line (SCC-4) secreted a soluble form of RANKL, and conditioned media from these cells stimulated osteoclastogenesis from precursors in a manner that was inhibited by an anti-RANKL antibody (25). Immunohistochemical analysis indicates that several other tumor types also express RANKL, including osteosarcomas, and both renal and lung metastases (47). Whether this RANKL expression has any pathological consequence has yet to be determined.

4. PHARMACOLOGIC EFFECTS OF RANKL ANTAGONISTS ON BONE METASTASES AND TUMOR-ASSOCIATED OSTEOLYSIS

At least three different classes of RANKL antagonists have been described: OPG, anti-RANKL antibodies, and a soluble non-signaling form of RANK (RANK-Fc). Each of these molecules acts by binding to RANKL and preventing it from interacting with RANK. RANKL antagonists have been used successfully in various animal models of bone metastasis and

tumor-associated osteolysis. In some models of osteolysis, such as humoral hypercalcemia of malignancy, the therapeutic effects of RANKL antagonists are essentially restricted to their suppressive effects on bone resorption. In models of experimental bone metastasis, RANKL antagonists also have the potential to directly or indirectly influence the behavior of tumor cells within the bone microenvironment. In animal studies, the inhibition of osteolysis by antiresorptive agents is often associated with a reduction in skeletal tumor growth (80, 81), while the stimulation of bone resorption has been shown to increase skeletal tumor growth (82). These and other observations provide support for Paget's "seed-and-soil hypothesis" (83), which posits that tumor cells (seeds) grow only when they localize in a target organ (soil) that provides them with a fertile environment. The high incidence and prevalence of bone metastasis suggests that the skeleton provides an especially fertile soil for malignancies such as breast, prostate and lung carcinomas (84).

This chapter has thus far described numerous mechanisms by which tumor cells influence the bone microenvironment through the OPG/RANK/RANKL pathway. This section summarizes the pharmacologic effects of RANKL antagonists in experimental models of bone metastasis. The majority of published studies suggest that the blockade of RANK/RANKL signaling makes bone a less fertile environment for tumor cells. Most of these studies used experimental models that bypass some of the important early events in the metastatic cascade. Despite this limitation, most of these models do recapitulate the critical interactions between bone and tumor cells and allow for some predictions on the effects of RANKL antagonism on tumor growth, tumor burden and tumor-associated osteolysis.

4.1 Humoral Hypercalcemia of Malignancy

HHM is a life-threatening condition that results from the elaboration of pro-resorptive cytokines from tumor cells that may or may not reside in bone. Most cases of HHM result from solid tumors that secrete factors such as PTHrP through the systemic circulation. Other cases occur in advanced stages of bone metastasis and with multiple myeloma, where a significant skeletal tumor burden is capable of inducing enough localized osteolysis to create hypercalcemia. Murine models of HHM have proven useful for determining the extent to which RANK/RANKL contributes to HHM. The precision and accuracy of serum and blood ionized calcium measurements have provided a sensitive measuring stick for evaluating the relative efficacy of different classes of antiresorptives.

The earliest description of OPG efficacy in an HHM model indicated that RANKL might represent a particularly vulnerable target for treating hypercalcemia. Nude mice were rendered hypercalcemic by the

transplantation of human pancreatic tumor tissue (85). OPG treatment of these mice resulted in the rapid (2 hour) and sustained (24 hour) reduction in serum calcium. The rapid kinetics of the calcium-lowering effect is consistent with the ability of OPG to inhibit the action of mature osteoclasts (19, 86). Such rapid calcium lowering effects have also been demonstrated with OPG treatment of thyroparathyroidectomized mice challenged with PTH 87. In a syngeneic tumor model of HHM, OPG was shown to both delay and reverse hypercalcemia (88). Colon adenocarcomina (C-26) cells were implanted subcutaneously into mice and hypercalcemia started to develop within nine days. When daily OPG treatments were initiated at this time, the development of hypercalcemia was prevented for the seven days of follow-up. In tumor-bearing mice that were allowed to become severely hypercalcemic prior to treatment, OPG completely reversed hypercalcemia within 48 hours (88).

Both of the tumors described above produce significant amounts of PTHrP, a factor that is thought to be responsible for most cases of HHM (reviewed in 89). PTHrP stimulates osteoclasts via the induction of RANKL and the suppression of OPG by osteoblasts and stromal cells (46). It is therefore not surprising that the addition of exogenous OPG has beneficial effects on HHM. PTHrP also promotes the renal reabsorption of calcium and increases intestinal calcium absorption (90), and these phenomena are likely to contribute to the challenge of controlling HHM. Currently approved antiresorptive agents such as pamidronate have a diminished therapeutic effect with repeated use (91), and it is possible that the actions of PTHrP outside of bone are responsible for this "resistance". OPG has a unique ability among antiresorptive agents to virtually eliminate osteoclasts in animals, and this property provides a useful tool for dissecting the relative contribution of osteoclasts to HHM. In the C-26 model of HHM, OPG treatment reduced osteoclast numbers to levels well below those observed in normal healthy mice. Dose response studies indicated that the control of hypercalcemia was directly related to the reduction in osteoclast numbers (88). In this model, osteoclasts appear to play a dominant role in mediating hypercalcemia.

The therapeutic significance of osteoclast reduction was further explored in a mouse model of PTHrP-mediated hypercalcemia. Mice were challenged twice daily with PTHrP, which caused hypercalcemia in association with increased osteoclast surfaces (92). OPG treatment rapidly (3 hours) and completely normalized serum calcium in hypercalcemic mice while reducing osteoclast numbers to sub-physiological levels. The bisphosphonate pamidronate, given at a maximum tolerated dose, did not reduce osteoclast numbers in PTHrP-challenged mice and had only modest calcium-lowering effects (92). OPG and pamidronate were also compared head-to-head in the

C-26 tumor model of HHM. OPG caused more rapid, more dramatic, and more prolonged reductions in serum calcium compared to pamidronate. OPG also caused significantly greater reductions in osteoclast surfaces compared to pamidronate (93). An interesting observation from these studies was that hypercalcemia eventually returned in OPG treated animals despite the persistence of profoundly reduced osteoclast surfaces (92, 93). It seems likely therefore that the early hypercalcemic response to PTHrP is mediated primarily by osteoclasts, while the chronic response involves progressively greater contributions from the gut and kidney. During the early resorption-mediated phase, the unique ability of OPG to eradicate osteoclasts may provide better control of hypercalcemia compared with some bisphosphonates.

4.2 Multiple Myeloma

Myeloma cells have a tendency to remain confined to the skeleton, where they induce bone resorption via the production or induction of RANKL. The dependence of myeloma cells on bone may be related to their complex interactions with bone marrow stromal cells (24). Marrow stromal cells can promote myeloma cell growth and survival 94. Marrow stromal cells are also probably required for the osteolysis associated with myeloma (23). In animal studies, myeloma cells cause osteolysis in association with significantly increased osteoclast numbers (24, 39). Recent evidence demonstrates that osteoclast activity may also be required for myeloma cell survival in bone (23, 24). This finding would predict that adequate control of osteoclasts might lead to better inhibition of myeloma growth in patients, while simultaneously protecting the skeleton. OPG completely eradicated osteoclasts and prevented osteolysis in mouse bones containing murine myeloma cells (39, 95). This therapeutic response was associated with a significant reduction in skeletal tumor burden and reduced morbidity (95).

These preclinical results provide a rationale for using RANKL antagonists to protect bone and reduce tumor burden in myltiple myeloma patients. OPG has recently been shown to have significant antiresorptive effects in patients with myeloma, but this study did not attempt to monitor tumor burden or disease progression (44). Mouse data has provided evidence that RANKL antagonists might have therapeutic benefits in myeloma beyond the expected bone-sparing effects. Human myeloma cells isolated from human bone marrow were injected intravenously into SCID mice bearing human bone xenografts (23). The myeloma cells colonized the bone xenografts and secreted detectable levels of human immunoglobulin into peripheral blood. The myeloma cells caused extensive osteolysis in association with elevated osteoclast numbers in the human bone. Treatment

of the mice with RANK-Fc caused a significant reduction in osteoclasts and protected bone from osteolysis. RANK-Fc also caused a marked reduction in serum levels of human immunoglobulin and also reduced skeletal tumor burden histologically (23). Immunohistochemistry indicated that myeloma cells induced an increase in RANKL and a reduction in OPG in the bone xenografts, and RANK-Fc treatment appeared to reverse these changes (23). These data highlight the dependence of human myeloma cells on the bone microenvironment. The ability of RANK-Fc to protect bone and reduce skeletal tumor burden in this myeloma model suggests that osteoclast activity is important for the growth and/or survival of myeloma cells in bone. The antagonism of RANKL may initiate a therapeutic cascade, wherein anti-osteoclast effects lead to myeloma cell growth inhibition, which prevents the myeloma cells from changing OPG/RANKL levels in a direction that would otherwise encourage further osteoclast activity.

4.3 Breast Cancer

The osteolytic nature of nearly all bone metastases from breast cancer provides a clear rationale for antiresorptive therapy. RANKL antagonists such as OPG may be particularly effective in this setting, due to the central role that RANKL in mediating osteolysis associated with breast cancer (48, 49). OPG can significantly reduce bone resorption in patients with breast cancer (44), but it remains to be determined whether this antiresorptive effect provides any therapeutic benefit. Meanwhile, animal studies with human MDA-231 breast cancer cells demonstrate that the antiresorptive effect of OPG is associated with significant benefits, including the preservation of bone and the suppression of skeletal tumor burden. MDA-231 cells are able to stimulate osteoclastogenesis and bone resorption (45). The intracardiac injection of human MDA-231 breast cancer cells in nude mice results in osteolytic bone metastases and increased osteoclast numbers. We examined the inhibitory effects of OPG on osteolysis and skeletal tumor burden using both treatment and prevention paradigms.

In the prevention paradigm, OPG or PBS (vehicle) treatment was initiated immediately after intracardiac tumor cell inoculation (81). Four weeks later, PBS treated animals had significant radiographically evident osteolytic disease. Histologic assessment revealed focal lytic lesions associated with numerous active osteoclasts. In mice treated with OPG there was no radiographic evidence of lytic disease. Histomorphometry revealed that OPG completely eliminated osteoclasts from tumor-bearing bones and also reduced skeletal tumor burden by 80% (81). In this prevention model, it is likely that the antiresorptive effects of OPG were occurring at the time when tumor cells were colonizing the skeleton. This experimental design

was suitable for testing the theory that an altered bone microenvironment can influence the progression of metastatic bone disease. However, results from this study might not predict the response to therapy when tumor cells have already metastasized to bone.

In clinical practice, breast cancer cells have often spread to bone at the time of initial diagnosis (96). Bone can be the first and only site for metastases (97), and bone-only metastases have a tendency to progress more slowly than soft tissue metastases (98). Thus there exists a significant window of opportunity for treating patients with established osteolytic disease. We tested the ability of OPG to prevent the progression of established osteolytic bone disease in the MDA-231 nude mouse model (99). Mice were inoculated with MDA-231 cells and allowed to develop radiographically evident osteolytic disease (3 weeks) before treatment was initiated.

Mice were divided into 3 groups such that each group had similar overall levels of osteolysis, and one group was sacrificed to serve as a baseline group. The other two groups were treated with either OPG or PBS for one week and were then sacrificed. During this final week, radiographic osteolysis increased by 400% in PBS-treated animals compared to the baseline group. In contrast, there was no increase in osteolysis in the OPG-treated group. Histologic assessment revealed a 210% increase in skeletal tumor burden in PBS-treated animals compared to baseline, whereas OPG treatment was associated with a non-significant 32% increase. OPG treatment also reduced osteoclast numbers by 96% compared to PBS treated mice. These data indicate that OPG treatment can rapidly and effectively control the growth and osteolysis of established bone metastases from human breast cancer cells, even when treatment is delayed until the tumor is undergoing rapid progression.

4.4 Prostate Cancer

Bone resorption is frequently elevated in prostate cancer patients with bone metastases (66, 67), and antiresorptives have been used to treat the osteolytic component of their lesions (63, 65). The exact mechanisms by which prostate cancer cells induce bone resorption is not clear, but recent data suggests that human prostate cancer cells can directly activate osteoclasts (16). Human C4-2B prostate cancer cells secreted a soluble form of RANKL, and conditioned medium from these cells was able to induce osteoclastogenesis in the absence of stromal cells. OPG blocked the osteoclastogenic effect of this conditioned medium. Injection of C4-2B cells into the tibiae of SCID mice resulted in increased osteoclast numbers and substantial osteolysis, and these responses were prevented when OPG

treatment was initiated at the time of tumor inoculation. While vehicle-treated mice developed significant skeletal tumor burden, no prostate tumor cells were evident in the bones of OPG-treated mice 4 weeks after tumor cell inoculation. OPG did not inhibit the growth of C4-2B cells grown subcutaneously or in culture, suggesting that the reduction in skeletal tumor burden was not due to direct effects of OPG on tumor cells (16). Similar results have been reported when human LuCaP prostate cancer cells are injected into the tibiae of nude mice (100). OPG treatment protected bones from osteolysis and also reduced skeletal tumor burden by 75-80%, as assessed by measuring human PSA levels in serum (100).

The mechanism by which OPG treatment reduced skeletal tumor burden is an important unanswered question. Skeletal tumor burden is a function of tumor cell colonization of bone and the subsequent growth and survival of those cells. In both of the model systems described above, a bolus of tumor cells is injected directly into bone. The absence of detectable tumor cells after four weeks in treated mice suggests that OPG treatment caused a reduction in tumor cell growth or survival in bone. The apparent lack of direct anti-tumor effects with OPG 16 suggests that any reduction in C4-2B tumor cell growth or survival was related to the antiresorptive effect of OPG. Bone may contain prostate cancer cell mitogens, as suggested by the observation that bone metastases from prostate cancer frequently had higher growth rates compared to the primary tumor (101). The identity of these mitogenic factors has not been determined, but TGF-β and IGF-I are conspicuous candidates. These growth factors are stored in bone matrix and both factors stimulate the proliferation of cultured prostate cancer cells (102-104). It was recently reported that IGF-I increases RANKL production by prostate cancer cells (105), a phenomenon that could create a positive feedback loop to promote further osteolysis and tumor cell growth. OPG can neutralize the RANKL ligand secreted by prostate cancer cells, and this action alone could account for the protection of bone and the reduction in skeletal tumor burden observed in these animal models.

4.5 Other Neoplasms

The available published literature indicates that RANKL antagonists can prevent osteolysis induced by a wide variety of tumor types. In many cases, the prevention of osteolysis with RANKL antagonists is associated with reductions in skeletal tumor burden as well. For example, the intracardiac injection of murine C-26 colon adenocarcinoma cells results in extensive osteolysis in association with increased osteoclasts. OPG treatment eliminated osteoclasts, prevented osteolysis, and significantly reduced skeletal tumor burden (81). Osteosarcoma cells also caused osteolysis and

increased osteoclast numbers when injected into the femurs of op/op mice, and OPG treatment reduced osteoclast numbers and skeletal tumor burden by over 90% (106). In these two models, OPG had no effect on tumor burden in non-osseous sites, suggesting that the anti-tumor effects observed in bone were mediated indirectly via the antiresorptive effect of OPG.

Bone pain from metastasis is a potentially debilitating and much feared consequence of disseminated disease. The pathways leading to pain are poorly understood, but recent animal data has provided novel insights that implicate osteoclast activity in this process. The injection osteolytic sarcoma cells into mouse femurs caused extensive bone destruction, bone pain, and pain-related changes in the neurochemical organization of the spinal cord. OPG treatment eliminated osteoclasts and prevented osteolysis, while also inhibiting the pain and pain-related neurochemical changes (107, 108). This study supports a role for osteoclast activity and/or bone resorption in the pathogenesis of bone pain. These data are the first to demonstrate that an antiresorptive agent can reverse the neurochemical changes associated with pain.

It is noteworthy that despite these pharmacologic benefits, OPG did not significantly reduce skeletal tumor burden in this model (107). This finding may be related to the type of tumor used and the route of administration. These tumor cells were directly inoculated into the femur, so it cannot be concluded that these tumor cells were in any way dependent on the bone microenvironment for their growth and/or survival. The lack of tumor burden suppression with OPG treatment could merely reflect the autonomous nature of these tumor cells in bone. In other words, not all osteolytic tumors depend on osteolysis for their growth or survival. For example, osteolytic Walker 256 carcinoma cells metastasize spontaneously to bone and cause extensive osteolysis (109). While the bisphosphonate pamidronate effectively blocked this osteolysis, skeletal tumor burden and tumor cell proliferation actually increased (110). Tumor inoculation into the heart or systemic circulation results in a more stochastic metastatic process, and bone metastases under these conditions are likely to be more dependent on the bone microenvironment. Whenever these routes of inoculation are used for osteolytic tumors, RANKL antagonism has consistently resulted in a decrease in skeletal tumor burden (23, 81, 99). It will be important to challenge these findings by testing a variety of RANKL antagonists in an expanded panel of metastatic tumor models. Head to head comparisons with other classes of antiresorptives will also be helpful to determine whether different mechanisms of antiresorptive action have differential effects on osteoclasts and on tumor burden.

5. CONCLUSIONS

Tumor-induced osteolysis is the major cause of morbidity in patients with bone metastases. Pain, hypercalcemia and pathologic fractures are common features of metastatic bone disease that could be adequately controlled with effective antiresorptive agents. The discovery of OPG/RANK/RANKL has provided a novel set of tools for studying the pathophysiological mechanisms responsible for osteoclast activation. Data reported so far indicates that RANKL is ultimately responsible for the bone resorption induced by virtually all tumors types. OPG, whether from endogenous sources or from pharmacologic administration, neutralizes RANKL and prevents it activating RANK on osteoclasts. Tumor cells have adopted several mechanisms for inducing bone resorption, and most of these mechanisms lead to an excess of RANKL relative to OPG. Tumor cells may produce their own RANKL, but it is more common for tumor cells to secrete factors that induce RANKL expression by host cells such as osteoblasts, stromal cells or T-cells. Tumor cells can also promote bone resorption by causing the suppression of OPG production by host cells. Multiple myeloma cells may also have the unique ability to directly bind and sequester OPG, thereby preventing it from neutralizing RANKL. In limited studies with cancer patients, OPG treatment causes rapid, significant and sustained reductions in markers of bone resorption. In animal models of experimental bone metastasis, OPG and other RANKL antagonists consistently reduce osteoclast numbers and prevent osteolysis. Protection of bone in these models is frequently associated with significant reductions in skeletal tumor burden. These observations provide a compelling rationale for testing the efficacy of RANKL antagonists in patients with bone metastasis.

LIST OF ABBREVIATIONS:

OPG:	Osteoprotegerin
RANK:	Receptor Activator of Nuclear Factor Kappa B
RANKL:	Receptor Activator of Nuclear Factor Kappa B Ligand
TNF-α:	Tumor Necrosis Factor Alpha
TR1:	TNF Receptor Superfamily Member 1
FDCR-1:	Follicular Dendritic Cell Receptor-1
OCIF:	Osteoclast Inhibitory Factor
KO:	Knockout
OPGL:	Osteoprotegerin Ligand
TRANCE:	TNF-Related Activation-Induced Cytokine
ODF:	Osteoclast Differentiation Factor

NF-κB:	Nuclear Factor Kappa B
JNK:	Jun N-Terminal Kinase
PTHrP:	Parathyroid Hormone Related Protein
$1,25(OH)_2D_3$:	1,25 Dihydroxyvitamin D_3
IL-1β:	Interleukin 1 Beta
IL-6:	Interleukin 6
WHO:	World Health Organization
mRNA:	Messenger Ribonucleic Acid
TGF-β:	Transforming Growth Factor Beta
ATL:	Adult T-Cell Leukemia
M-CSF:	Macrophage Colony Stimulating Factor
C-26:	Colon 26 Adenocarcinoma
SCID:	Severe Combined Immunodeficiency
PSA:	Prostate Specific Antigen
IGF-I:	Insulin-Like Growth Factor I
HHM:	Humoral Hypercalcemia Of Malignancy

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Chapter 12

ROLE OF MATRIX METALLOPROTEINASES IN BONE METASTASIS FROM HUMAN BREAST AND PROSTATE CANCER

Wilhelmina C.M. Duivenvoorden* and Gurmit Singh
Juravinski Cancer Centre, McMaster University, Hamilton Ontario, Canada
**Current address: Northwestern Ontario Regional Cancer Centre, Thunder Bay Ontario, Canada*

1. INCIDENCE AND MANIFESTATION OF BONE METASTASIS

Bone metastases are a significant problem in patients with cancer of the breast and prostate. Bone metastases are associated with extensive morbidity and mortality. They cause clinical disease due to extensive, intractable bone pain, pathological fractures, and spinal cord compression and leads eventually to death. 70% of patients that die of breast cancer have noticeable disease in their bones (1, 2). Similar numbers are found for prostate cancer patients (2). Bone disease is also a major problem in multiple myeloma patients. The manifestation of the bone involvement is distinct for the three types of cancers.

Bone metastasis from breast carcinoma is frequently associated with osteolysis leading to extensive morbidity from bone pain, fractures and hypercalcaemia (3). Data from the MD Anderson Cancer Centre show that out of the 1171 patients that were entered on treatment for metastatic breast cancer, two thirds had involvement of bone. 180 of these patients had metastasis in bone only, of those 48% had osteolytic disease, 13% pure osteoblastic and the remaining presented with mixed metastasis. The most common skeletal areas involved are the pelvis and lumbosacral spine (4).

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Biochemical markers for bone metastasis in breast cancer patients are increased serum alkaline phosphatase, a marker of bone deposition, and urinary galactosyl-hydroxylysine, a marker of collagen catabolism (5). Tumor-associated hypercalcaemia is observed in 30% of breast cancer patients with bone involvement (6).

Prostate cancer patients exhibit primarily osteoblastic or osteosclerotic bone disease. The presence of tumor cells in the bone causes stimulation of osteoblastic activity resulting in increased bone mass adjacent to the tumor cells (7). However, increased bone resorption has also been documented histologically and biochemically in prostate cancer patients (8). Metabolic changes that are associated with bone metastasis in prostate cancer are hypocalcaemia and increased serum bone alkaline phosphatase as a marker of osteoblastic activity. More surprisingly, increased urinary deoxypyridoline and pyridinoline cross-linked carboxyterminal telopeptide of type I collagen, which are both markers of osteoclastic activity, have been observed in prostate cancer patients with metastatic bone disease (9).

Multiple myeloma is a malignant neoplasm resulting from monoclonal proliferation of plasma cells, which is associated with the overproduction of monoclonal IgG, IgA and/or light chains. The clinical presentation of the disease is often characterized by bone pain as a result of very extensive bone destruction. As a consequence of the myeloma cell infiltration an increase in osteoclastic activity and proliferation occurs mediated by the release of osteoclast-activating factors produced locally in the bone microenvironment by cells of both tumor and non-tumor origin (10). In about 30% of all multiple myeloma patients tumor-associated hypercalcaemia is observed (6).

2. MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are a large class of closely related collagenases involved in degradation and remodelling of the extracellular matrix in embryogenesis and tissue repair [recently reviewed in (11, 12)]. The activity of MMPs is tightly regulated in physiological situations where extracellular remodelling must occur, e.g., in morphogenesis and wound healing. The enzymes can be divided into six broad classes on the basis of sequence homology and substrate specificity (Table 1). Collectively, MMPs are capable of degrading all of the components of the extracellular matrix (13, 14). One class of MMPs consists of the membrane-bound MMPs. MT1-MMP, MT2-MMP, MT3-MMP and MT5-MMP all contain a transmembrane domain, which facilitates the cell-mediated activation of 72-kDa type IV collagenase (MMP-2) (15-20), whereas MT4-MMP and MT6-MMP are attached to the membrane through a glycosylphosphatidylinositol (GPI)-

anchor. The more recently discovered MMP-23 contains a cysteine array and a unique interleukin-1 receptor-related domain (21). It is a type II transmembrane MMP with an N-terminal signal anchor (22).

Table 1. Human family of matrix metalloproteinases and their classical substrates

Class of MMPs	MMP	Substrate
Interstitial collagenases	Interstitial collagenase (MMP-1)	Type I, II, and III fibrillar collagens
	Neutrophil collagenase (MMP-8)	
	Collagenase-3 (MMP-13)	
Stromelysins	Stromelysin-1 (MMP-3)	Proteoglycans, laminin, type IV collagen and type X collagen
	Stromelysin-2 (MMP-10)	
Matrilysins	Matrilysin (MMP-7)	Fibronectin, laminin, type IV and X collagen
	Matrilysin-2 (MMP-26)	-
Type IV collagenases	72-kDa type IV collagenase (MMP-2)	Type IV and type V non-fibrillar collagens, type VII collagen, fibronectin, type X collagen, elastin
	92-kDa type IV collagenase (MMP-9)	
Furin-activatable MMPs	Stromelysin-3 (MMP-11)	α1-proteinase inhibitor
	Epilysin (MMP-28)	Casein
Membrane type MMPs	MT1-MMP (MMP-14)	
	MT2-MMP (MMP-15)	Latent MMP-2
	MT3-MMP (MMP-16)	
	MT4-MMP (MMP-17)	-
	MT5-MMP (MMP-24)	Latent MMP-2
	MT6-MMP (MMP-25, Leukolysin)	Collagen IV
Type II transmembrane MMPs	MMP-23	
Other MMPs	Metalloelastase (MMP-12)	Elastin, type IV collagen, laminin, fibronectin
	MMP-19	Type IV collagen, laminin, fibronectin, aggrecan
	Enamelysin (MMP-20)	Amelogenin, type XVIII collagen
	MMP-27	-

2.1 Regulation of MMP Expression and Activity

Matrix metalloproteinase activity is tightly regulated and occurs at different levels. Regulation of gene expression occurs at both the transcriptional and posttranscriptional level. AP-1 and ETS binding sites are among the most important *cis*-acting elements found in the promoter regions

of the majority of the MMP genes, except for MMP-2. They are involved in the stimulation of MMP gene expression by phorbol esters, serum, and IL-1 (23, 24). Epidermal growth factor (EGF) has been shown to increase the transcription of interstitial collagenase (MMP-1) in human breast adenocarcinoma MDA-MB-231 cells (25). Transforming growth factor-β1 (TGF-β1) and retinoic acid inhibit the expression of MMP-1 at the transcriptional level (26-28). In the repression by TGF-β1, the AP-1 site cooperates with the TIE (transforming growth factor inhibitor element) site (29), whereas more distal sites of the promoter of MMP-1 are involved in the repression by all-*trans*-retinoic acid (27).

MMPs are secreted as latent enzymes that have to be proteolytically modified for activation. They are activated by removal of a portion of the amino-terminal part of the enzyme (30). *In vivo* activation occurs through a cascade of activation of various latent proteinases. Serine proteases such as trypsin can activate various latent MMPs. Another serine protease, plasmin, can be both directly involved in the degradation of collagen I as well as activate latent MMP-1 and stromelysin-1 (MMP-3) (31). Plasmin in turn is generated from plasminogen through the action of predominantly the urokinase-type plasminogen activator cascade (32). Some members of the MMP family can directly activate other MMPs. For example, collagenase-3 (MMP-13) can be activated by MT1-MMP, MMP-3 and MMP-2 (33, 34). MMP-3 can carry out the final activation step of MMP-1 and MMP-3 after they have been cleaved by plasmin or trypsin. MMP-3 can also completely activate latent 92-kDa type IV collagenase (MMP-9) (35).

MMP-2 is in several regards distinct from the other members of the MMP family. Firstly, it is not activated in a similar fashion as the other MMPs. MMP-2 cannot be activated by plasmin, trypsin, chymotrypsin, thrombin, cathepsin G or MMP-3 (36), but is activated through a mechanism involving membrane-bound proteins. As recently as 1991 Ward *et al.* reported the activation of latent MMP-2 by plasma membranes isolated from fibroblasts (37). Using degenerate primers homologous to the conserved sequences in the cysteine switch region and the catalytic domain of MMPs led to the cloning of the first membrane-type MMP, MT1-MMP. Expression of this protein in COS-1 cells shows its presence on the cell surface (38).

Tissue inhibitors of metalloproteinases (TIMPs) are the natural endogenous inhibitors of MMPs. TIMPs are small proteins that non-covalently form 1:1 complexes with specific MMPs, blocking their proteolytic effect (39). TIMP-1 specifically binds to most MMP family members, whereas TIMP-2 binds and inhibits MMP-2. This can occur through two distinct mechanisms; binding to proMMP-2 and thereby inhibiting the cleavage into active MMP-2 or by binding to MMP-2 and inhibiting its activity (37). TIMP-3 is associated with the extracellular matrix

and not soluble like the other three TIMPs (40). The latest member of the TIMP family, TIMP-4, was cloned in 1996 from a heart cDNA library (42). TIMP-3 and TIMP-1 are equally effective in inhibiting MMP-1, MMP-2, MMP-3 and 92-kDa type IV collagenase (MMP-9) (41). A balance between MMPs and TIMPs determines the overall collagenolytic activity.

Unlike other MMPs, MMP-2 is usually found complexed with TIMP-2 through their respective C-terminal domains. A second binding site for TIMP-2 on the N-terminal part of the MMP-2 distinct from the active site also exists (43). Ultimately the balance of TIMP-2 over MMP-2 will decide whether MMP-2 is activated by MT1-MMP. TIMP-2 actually has a dual effect on the activation of proMMP-2; at low concentrations TIMP-2 enhances MMP-2 activation, whereas at high concentrations it acts inhibitory. When the ratio of TIMP-2:MMP-2 is between 1 and 2, TIMP-2 through its N-terminal domain binds to MT1-MMP and through its C-terminal end binds to the C-terminal domain of proMMP-2, which can subsequently be presented to and activated by a neighbouring MT1-MMP molecule. The MT1-MMP molecule does not necessarily have to be located on the same cell. If the ratio is greater than 2 there is enough TIMP-2 to bind to proMMP-2 and, separately, to MT1-MMP. This therefore does not lead to activation (44).

Lastly, MMPs are inactivated through degradation. Plasmin, which is involved in the activation of MMPs, can also play, under other circumstances, a role in the degradation process of MMPs (45). In the presence of plasmin, uncomplexed MMP-2 is degraded, whereas the binding of TIMP-2 to MMP-2 prevents the degradation by plasmin (46, 47). Interaction of MMP-2 and MMP-9 with the cell surface may also partially protect the MMPs from degradation (46). MT1-MMP degradation into an inactive 43 kDa form occurs within 1-4 h after translation, which parallels the activation of MMP-2. Interestingly, this process is inhibitable by a synthetic MMP inhibitor (45). Stromelysin-3 (MMP-11) is inactivated in a similar MMP-dependent manner (48).

2.2 Non-Classical MMP Substrates

More recently novel MMP substrates other than components of the extracellular matrix have been discovered. The ability of MMPs to cleave growth factors, proteins involved in cell adhesion, and growth factor receptors suggests different mechanisms of action for MMPs in the development of metastatic cancer.

MMP-2 and MMP-9 can release TGF-β from its latent complex (49). Tumor necrosis factor-α (TNF-α), a potent pro-inflammatory cytokine, is expressed as a 26-kDa precursor found anchored on the membrane of

leukocytes. In order for TNF-α to become biologically active it has to be proteolytically cleaved. Synthetic MMP inhibitors have been found to specifically inhibit this process. Matrilysin (MMP-7), stromelysin-1 (MMP-3), MMP-1, and to a slightly lesser extent MMP-2 and MMP-9 are capable of releasing TNF-α (50). Processing of the precursor of IL-1β into its biologically active form can be carried out by MMP-3 and MMP-9 and, to a lesser extent, MMP-2, but not by MMP-1. This process is independent of caspase-1 (IL-1β-converting enzyme) (51).

Proteins involved in cell adhesion, such as CD44, E-cadherin and laminin, are also substrates for MMPs. MT1-MMP has been shown to cleave the ecto-domain of the CD44 receptor (52). CD44 is the main cell surface receptor for hyaluronate, a component of the extracellular matrix. Treatment with 1,10-phenanthroline, a general MMP-inhibitor, prevents cleavage and results in the suppression of lung cancer cell migration on hyaluronate as a substrate (53). Upon cleavage of E-cadherin by MMP-7 and MMP-3, the released E-cadherin fragment can promote invasion (54). Laminin-5 can be cleaved by MMP-2, which results in the exposure of a cryptic site on laminin-5 that induces cell motility (55).

MMPs are involved in the shedding of the HER2/*neu* receptor, the receptor that is frequently overexpressed in human breast cancer. TIMP-1, but not TIMP-2, EDTA and a synthetic MMP inhibitor, batimastat, can inhibit the shedding. Cleavage of the extracellular domain of the HER2/*neu* receptor is associated with poor prognosis and resistance to chemotherapy and endocrine therapy in breast cancer, since this truncated HER2 protein has the capacity to enhance signalling (56). The shedding of the interleukin-6 receptor observed in multiple myeloma can also be inhibited by MMP-inhibitors (TIMP-3 and batimastat, but not TIMP-1 or -2) (57). Similarly, TIMP-3-overexpressing colon carcinoma cells show increased sensitivity towards apoptosis induced by serum starvation or hydrogen peroxide treatment probably through stabilization of TNF-α receptors on the cells' surface (58).

Metalloelastase (MMP-12) cleaves plasminogen to generate angiostatin, a protein with anti-angiogenic properties. In hepatocellular carcinoma patients undergoing curative partial hepatectomy a significant association between MMP-12 mRNA and angiostatin protein is reported with angiostatin found in the tumorous tissue and a correlation with survival (59). Such non-classical properties offer an additional role for MMPs in tumor progression.

2.3 MMP Expression and Activity in Cancer

Matrix metalloproteinases are involved in various steps of the development of metastasis, since they are able to degrade the extracellular matrix and the components of basement membranes, which underlie the endothelial cells. They can thus facilitate access to blood vessels and allow the development of tumor growth at distant sites (31, 60). There is substantial evidence that a positive correlation between the expression of MMPs and the invasiveness and/or grade of the tumor exists. Animal and *in vitro* experiments have provided extensive evidence to support the role of MMPs in the process of invasion and metastasis. A direct correlation between the lung metastatic potential of cancer cells and the capacity to degrade type IV collagen was first reported in 1980 (61). Those initial findings have been confirmed in other experiments using differentially metastatic clones of rat mammary adenocarcinoma cells (62) and cell hybrids made from cells with different metastatic potential (63). In oncogene-transformed rat cell lines with different metastatic properties the levels of the stromelysin-1 and -2 (MMP-3 and MMP-10), correlate positively with the metastatic phenotype of the cells, whereas the expression of MMP-2 and MMP-9 is not associated with metastasis in these cell lines (64). Data from gene-knockout studies in animals underline the importance of the role of MMPs in tumor and metastasis development. A strain of mice prone to develop intestinal adenoma that is also deficient for MMP-7 shows a 58% reduction in the amount of tumors compared to MMP-7 +/+ mice (65). Stromelysin-3 (MMP-11)-deficient mice demonstrate a reduction in DMBA-induced carcinoma incidence and tumor size (66). In MMP-9-deficient mice the number of metastatic colonies from B16/BL6 and Lewis lung carcinoma decreases by 45 and 59% respectively (67).

To date, a correlation between elevated MMP expression and the stage of the disease, diagnosis or survival has been reported for many different types of human cancers. Elevation of MMP-9 has been documented in breast (68), pancreatic (69), prostate (70, 71), ovarian (72, 73), and bladder cancer (74), lung tumors, squamous cell carcinoma of the uterine cervix (75), and also hepatocellular carcinoma (76). Other MMPs have been associated with tumor progression as well. High levels of MMP-1 protein (77) and increased collagenolytic activity have been observed in colorectal cancer (78, 79). MMP-1 has also been found overexpressed in head and neck squamous cell carcinomas. In these cancers this coincided with an overexpression of MMP-10 (80). In human breast adenocarcinomas MMP-1 immunoreactivity correlates inversely with stage and progesterone receptor status of the cancer, however, only when the immunoreactivity found in the stromal cells, but not in cancer cells is considered (81). Another collagenase, collagenase-3

or MMP-13, originally cloned from breast cancer tissue (82) has been localized to tumor cells in squamous cell carcinoma of the head and neck (83) and invasive vulvar squamous cell carcinomas (84). In breast carcinoma, an inverse correlation between MMP-11 and long-term survival has been observed both at the level of mRNA expression (in 55 patients with invasive breast cancer) (85) and the level of protein expression (in 111 patients) (86). MMP-7 expression is confined to samples of prostate cancer tissue, and is hardly detectable in normal or hyperplastic prostate tissue (87). MMP-2 expression has been correlated to invasiveness in ovarian cancer (88, 89).

MMP-2 has been found overexpressed on the mRNA level in pancreatic (69), prostate (90) and colorectal cancer (91). Active MMP-2, but not latent MMP-2, has been found to correlate with invasiveness in bladder cancer (74) and with tumor grade in malignant breast cancer (68). An elevation of MT1-MMP and the association with an increased ratio of active MMP-2 over latent MMP-2 has been reported in several cancers. The expression of MT1-MMP in breast adenocarcinoma correlates with lymph node and distant metastasis and clinical stage of the tumor. This coincides with an increased activation of proMMP-2 into active MMP-2 and correlates with MT1-MMP, but not MT2-MMP or MT3-MMP mRNA expression (92). Similar results were found for papillary thyroid carcinomas (93), gastric carcinoma (94-96), brain tumors, including astrocytomas and glioblastomas (97), and hepatocellular carcinomas (98, 99).

In squamous cell carcinoma of the uterine cervix, the ratio of MMP-2+MMP-9 over TIMP-1+TIMP-2 has been determined. A high ratio of MMP:TIMP is associated with a poor prognosis (100). Similarly, in pancreatic tumors the mRNA of MMP-2 and MMP-9 is elevated compared to control tissue, and the cancerous tissue shows a concomitant increase in the levels of TIMP-1 and TIMP-2 (69). No ratio was determined in this study. Many of these data are derived from studies using *in situ* hybridization or RT-PCR, which can only detect quantity and location of the transcript or Northern blotting which can only be employed semi-quantitatively. Immunohistochemistry, which has also been widely used, localizes specific proteins, but is limited through the sensitivity and specificity of the antibodies used. A true measure that takes into account the amount of active protein, the availability, and binding to TIMPs, is *in situ* enzymography. However, this technique is limited to only a few substrates (gelatin, casein) and will not distinguish between different MMPs that cleave the same substrate. It has been employed in an elegant study looking at human papillary thyroid carcinoma. The authors performed a thorough study to establish a relationship between MT1-MMP expression and active MMP-2. Normal control, follicular adenoma and carcinoma tissue homogenates

were used for ELISA, gelatin enzymography and Northern blot analysis for MT1-MMPs and showed a strong correlation between the stage of the disease and the level of activation of MMP-2. Levels of MT1-MMP as determined by Northern blot correlated with the activation of MMP-2 and also with lymph node metastases. *In situ* hybridization and immunohistochemistry showed that MT1-MMP, MT2-MMP and MMP-2 are expressed by both tumor and stromal cells, however, *in situ* enzymography revealed that the activity is predominantly present in the carcinoma cells (93).

2.4 TIMP Expression in Cancer

As stated before, the overall collagenolytic activity of cancer cells is determined by a balance between active enzymes and TIMPs. Classical experimental studies have reported that overexpression or addition of recombinant TIMP in metastatic cells is associated with an inhibition of invasive and metastatic potential *in vitro* and *in vivo* (101-104) and neovascularization can be blocked (105). TIMP-1, -2 or -3 gene delivery using adenovirus techniques in two metastatic melanoma cell lines can inhibit invasion through a reconstituted basement membrane with TIMP-3 overexpression being the most effective (106). TIMP-3- or TIMP-4-transfected human mammary adenocarcinoma MDA-MB-435 cells show significantly slower tumor growth *in vivo* compared to control cells (107, 108). Injection of TIMP-2-transfected breast cancer MDA-MB-231 cells into nude mice results in significantly less radiologically detectable osteolytic bone tumors and coincides with an increased survival of the mice (109). When taking into consideration both MMPs and TIMPs, highly metastatic clones of human prostate cancer PC-3 cells have been found to contain relatively high levels of MMP-2 and low amounts of TIMP-2 when compared to the less invasive clones (110). Reduced levels of free TIMP are associated with higher levels of active MMP-2 in malignant explant prostate cultures when compared to normal and benign prostate samples (111). Samples obtained from gastric cancer patients dying from the disease are more likely to have a lower percentage of TIMP-2 expressing cells and high levels of MMP-2 expressing cells (112). Immunolabelling of gastric cancer tissue revealed a correlation between TIMP-2 staining and metastasis. Among advanced gastric carcinomas cases neoplasms with diffuse and strong staining for TIMP-2 have less frequently nodal metastasis (29%) when compared to the neoplasms that show focal and weak TIMP-2 staining (69% of these cases have metastasis) (113). A comparison of cutaneous basal and squamous cell carcinoma revealed significantly higher levels of TIMP-2 mRNA in the cutaneous basal carcinoma tissue samples, suggesting

an inverse correlation between TIMP-2 expression and the metastatic capacity of these tumors (114). Kikkawa et al. (115) employed enzymography and reverse enzymography to ovarian cancer samples and found that high levels of TIMP-1 as well as MMP-9 are related to the malignant phenotype.

However, not only have increased MMP expression and concomitant decreased TIMP expression been reported in several cancers (69, 100, 112, 115), overexpression of TIMP has also been associated with human cancers. In thyroid carcinoma TIMP-1 mRNA levels correlate directly with tumor aggressiveness (116). High levels of TIMP-1 mRNA significantly correlate with the presence of lymph node metastases in breast cancer (117) and colorectal cancer (118), whereas elevated expression of TIMP-2 is associated with development of distant metastases in breast cancer (117) and with tumor stage in colorectal cancer (83). On the protein level, elevated levels of TIMP-1 have been found in colon cancer samples compared to paired samples of adjacent mucosa (119). Increased TIMP-1 expression predicts shortened survival in renal cell carcinoma patients (120). Unfortunately, most studies, with the exception of one, make no statements with regard to the status of the various MMPs. A colorectal cancer study (212 cases) employed immunohistochemistry to describe the staining of tumor cells for MMP-2 and of infiltrating macrophages for MMP-9. However, neither MMP could be used as a marker for staging or prognosis of the disease. TIMP-2-staining of the basement membrane and the interstitial stroma, but not of the epithelium, is positively correlated to Dukes' stages (83). On first glance these findings defy the concept of the involvement of MMPs and TIMPs in cancer invasion and metastasis. However, it has been reported since then that binding of TIMP-2 to MMP-2 can prevent the degradation of MMP-2 by plasmin, whereas in the presence of soluble plasmin, uncomplexed MMP-2 is degraded (46). In addition, a more complex role of TIMPs independently of MMPs has since been recognized. TIMP-1 is known to possess erythroid-potentiating activity (EPA) and can function as a mitogen in a variety of cell lines (121) and similar properties have been accounted to TIMP-2 (122). Overexpression of TIMP-1 and/or -2 can confer resistance to induced apoptosis in B-cells, breast epithelial and melanoma cells (123-125). An increase in TIMP could also represent a defense mechanism by the stromal compartment of the host tissue (126).

3. REMODELLING IN BONE AND BONE METASTASIS

Bone is a specialized connective tissue that consists of a two-componential matrix. The organic, unmineralized matrix also called osteoid is predominantly composed of type I collagen and small amounts of proteoglycan, lipids, fibronectin and osteonectin. The inorganic mineral phase consists of hydroxyapatite crystals and makes up two thirds of the total matrix volume. Throughout life, the bone mass is continuously remodelled. In adults between the ages 20 and 30, 15% of the total bone mass turns over every year. Bone is remodelled by a concerted action of both the osteoblasts, responsible for the synthesis of organic matrix and calcification, and the osteoclasts, multinucleated cells of haematopoietic origin, responsible for bone resorption (127). During resorption, the osteoclasts create a sealed resorption compartment on the bone surface; the membrane of the osteoclast becomes heavily folded to increase the surface for lytic activity, creating the so-called ruffled border. Acid and proteolytic enzymes are secreted into this extracellular compartment and mediate the degradation of both the organic and inorganic components of the bone. The activity of osteoblasts and osteoclasts are regulated in a coordinated manner to maintain a balance between phases of osteoblastic bone formation and osteoclastic bone resorption. The resorption cavity created by the osteoclast is often the site of subsequent osteoblastic activity to fill the resorbed areas with new bone (127).

The spread of breast cancer cells to bone is typically accompanied by extensive osteolysis of mineralized collagenous bone matrix. While the mechanisms responsible for this pathologic bone loss are poorly understood, it has been demonstrated that the proteolytic degradation of the organic bone matrix is necessary and sufficient to activate osteoclastic bone resorption (128, 129). Therefore the degradation of type I collagen by MMPs is thought to be an essential initiating and propagating step in bone resorption.

Not only osteoclasts, but also tumor cells and tumor-associated macrophages have been identified as mediators of metastasis-associated osteolysis (6, 130). Bone resorption increases progressively from bone distant from metastatic breast tumor cells to bone right at the site of the tumor. Immediately adjacent to the tumor and within the metastases the number of osteoclasts is significantly increased. This suggests that increased resorption in breast cancer is mainly osteoclast mediated and locally activated by the tumor (131). In multiple myeloma the osteoclasts appear to accumulate on the bone-resorbing surfaces adjacent to collections of myeloma cells. Measurement of bone resorption using VX2 carcinoma cells injected into rabbit tibiae has shown that in the initial stages of invasion

osteoclastic bone degradation is more prominent, while in the later stages of bone destruction the tumor cells seem to mediate the bone resorption. At the later stages the osteoclasts are absent. Human tumor samples from various malignant diseases similarly show these two distinct phases of bone resorption (132). Tumor-associated macrophages isolated from breast cancer tissue have the potential to differentiate into TRAP (tartrate-resistant acid phosphatase)-positive multinucleated cells when co-cultured with osteoblast-like cells (133). These osteoclast-like multinucleated cells can resorb bone, however without the presence of the ruffled border structure that is characteristic for osteoclasts (130). It has been previously shown in our laboratory that bone metastatic cancer cells such as human prostate adenocarcinoma PC-3 cells can directly degrade osteoid-like extracellular matrices and fetal rat calvarial bone *in vitro* and that MMPs are involved in the process (134). All these tumor and tumor-associated cell types possess the potential to degrade collagenous matrix and thereby expose bone mineral, which may stimulate osteoclastic bone resorption.

3.1 MMP Expression in Bone and Bone Metastasis

In addition to cysteine proteinases, especially cathepsin K (135, 136), MMPs are believed to contribute to the physiological bone matrix degradation. MMP-1 is the most important among the MMP family members, since it specifically degrades native type I collagen, the protein most abundantly present in the organic matrix of the bone. More recently another interstitial collagenase, MMP-13, has been found to be able to degrade native type I collagen. However, the role of MMP-13 appears less significant in bone resorption, since it more efficiently degrades type II collagen. MMP-13 can be immunolocalized in prepubertal bone to osteoblasts only, and is absent from the epiphyseal area of adult bone. MMP-13 might therefore be more important during primary and secondary epiphyseal ossification (137). MMP-2, although to a lesser extent, is also able to degrade native type I collagen (31), whereas MMP-2 and MMP-9 (138, 139) are known to attack degraded type I collagen.

MMP-1, MMP-2, MMP-9 and both TIMP-1 and -2 have been identified in cultured human osteoblasts (140). In human bone tissue samples MMP-1 has been immunolocalized to both the lining osteoblasts and the cells synthesizing matrix (141). Immunohistochemistry experiments also show that MMP-1 is present in osteoblasts to a much higher extent than in the osteoclasts. This lends support to the hypothesis that osteoblast-derived MMPs play a role in the removal of surface collagenous osteoid, which precedes the osteoclast attachment and the actual bone resorption. *In situ* hybridization, using antisense MMP-9, shows that MMP-9 localizes to the

osteoclasts, whereas osteoblasts show only very little MMP-9 staining and not in all cases (142). The MMPs secreted by the osteoclast, the only cell in the bone with the definitive ability to degrade mineralized matrix, are unlikely to be involved in the initial stages of resorption in which bone mineral is dissolved in a highly acidic environment. However, at a later stage when the subcellular zone approaches neutral pH, MMPs may be secreted by the osteoclasts in order to degrade unmineralized matrix.

In an elaborate immunohistochemical study we compared the expression of MMP-1, MMP-2, MMP-9, MT1 MMP and TIMP-1 and TIMP-2 in 31 archival cases of bone metastasis from breast carcinoma to the expression in specimens of primary breast cancer, lymph nodes (from breast cancer patients), and normal breast (143). In tumor-associated fibroblasts TIMP-1 expression is increased in bone metastasis specimens when compared with primary and lymph node tumors. For the MMPs, no major differences between bone metastases and primary or lymph node tumors are found. MMP-1, MT1-MMP and TIMP-1 staining is observed in tumor cells, while MMP-1, MMP-2 and TIMP-2 staining is typically seen in fibroblasts associated with the tumor. The expression of both MMP-2 and MMP-9 is markedly increased in desmoplastic fibroblasts in primary and metastatic tumors as compared to fibroblasts in normal breast tissue. The expression and localization of MMP-1, MMP-9 and TIMP-1 in bone cells in metastatic bone corresponds to what has been described in normal bone suggesting that the expression of these enzymes is not affected by the presence of tumor. However, and not surprisingly, the number and activity of both osteoblasts and osteoclasts is greatly increased in bone metastatic samples (143).

3.2 Expression of MMPs as a Result of Tumor Cell-Invasion

Studies have shown that the stromal expression of numerous MMPs is frequently more prominent than expression by tumor cells themselves. In addition the pattern of MMP expression is tissue-specific. In breast cancer, where many different members of the MMP family are expressed, MMP-1, MMP-3, MT1-MMP and MMP-2 are found in the stromal cells at the mRNA level (81, 144-149). In contrast, by immunohistochemistry MMP-1 and MMP-3 protein are detected predominantly in cancer cells, and only in some cases in stromal cells (81). MMP-2 protein is also found localized in tumor cells (148, 150, 151). Similarly, MT1-MMP protein is localized to the tumor cells in one study (92), but to tumor cells and stromal cells in another (146). MMP-11 has been localized to the stromal cells both by *in situ* hybridization and immunohistochemistry (85, 86, 152). MMP-13, on the mRNA and protein level, is confined to the tumor cells (82). MMP-9 has been

demonstrated to localize to macrophages, smooth muscle cells and neutrophils (153). In other studies MMP-9 has been localized to tumor cells, both by *in situ* hybridization (68) and immunohistochemically (151). Immunohistochemical localization of MMPs has been problematic. Some studies localize a certain MMP to fibroblasts, while others describe the same MMP localized to tumor cells, depending on the antibody used. In addition, the results sometimes contradict the findings of *in situ* hybridization experiments.

Other cancers that are associated with bone metastases have been less extensively studied. In prostate carcinoma tissue MMP-2 is expressed by the epithelial neoplastic cells (70, 90, 154) with very little MMP-2 immunostaining in the stromal cells, and only in prostate adenocarcinoma and not in hyperplastic or normal prostate tissue (70). Immunohistochemistry has demonstrated MMP-9 antigenicity in both stromal and epithelial structures of the majority of prostate carcinoma specimens (155). *In situ* hybridization of prostate cancer tissue shows expression of MMP-7 in epithelial cells only and not in the stroma (87). In multiple myeloma the presence of the malignant cells in the bone has a very profound effect on the host tissue. MMP-2 and MMP-9 activity is found in the myeloma cells, whereas the myeloma cells induce MMP-1 expression in the bone marrow stromal cells. MMP-2 activity contributed by the stromal cells is activated in the presence of myeloma cells (156). The tissue- and cell-specific expression of MMPs might contribute to the different characteristics of bone metastasis from various types of primary cancers.

The spread of breast tumor cells to bone is typically accompanied by extensive osteolysis of mineralized collagenous bone matrix. Human breast cancer cells injected into the femora of mice induce an increase in osteoclast number at sites of tumor osteolysis. Conditioned medium derived from the cancer cells has an analogous effect *in vitro* (157, 158). In prostate cancer the presence of tumor cells in the bone stimulates osteoblastic activity resulting in increased bone mass adjacent to the tumor cells. Reciprocal interactions between prostate cancer cells and bone stroma appear to account for the typical osteoblastic response (159-161).

Degradation of collagenous bone matrix in bone metastasis may be the result of increased MMP expression that can occur via several different mechanisms. Tumor cells themselves secrete MMPs (162, 163) that are potentially upregulated during metastasis. Tumor cells also produce cytokines and growth factors that can affect the MMP expression in stromal cells. Conditioned medium derived from human breast adenocarcinoma H-31 cells stimulates the degradation of type I collagen by mouse osteoblast-like cells and bone resorption *in vitro* (164). Human breast cancer cells express and produce interleukin-6 as well as interleukin-11, both potent

stimulators of osteoclast development (165). Other factors that could be specifically involved in the upregulation of MMP mRNA expression are interleukin-1 and TNF-α (24, 156). Endothelial cell monolayers have been shown to become increasingly permeable when incubated with cancer cell-conditioned media, an effect inhibitable by 1,10-phenanthroline (166).

Membrane-bound proteins, such as EMMPRIN (Extracellular Matrix MetalloProteinase Inducer), are also involved in the induction of MMP expression in stromal cells. EMMPRIN is highly enriched around the cell's surface and present in tumor cells of lung and breast cancer, but not in normal tissue surrounding the tumor or non-cancerous tissue (167). EMMPRIN isolated from lung carcinoma LX-1 cells has been shown to stimulate production of MMP-1 in fibroblasts (168).

We applied an *in vitro* model system to study the effect of bone-metastatic tumor cells on MMP expression in different human cell lines representative of cell populations present in the bone. We used two types of co-culture systems with the following human cell lines, (i) osteoblast-like cells Saos-2 (169), (ii) GCT, a cell line of monocytic origin (170) with many properties of osteoclasts including TRAP-activity and CD68 antigen expression, (iii) HUV-EC-C, primary endothelial cells derived from a umbilical cord vein (171), and (iv) fibroblast cells (Hf-172). Firstly, we used a co-culture consisting of a monolayer of bone-type cells with human bone-metastatic cancer cells (human mammary adenocarcinoma MDA-MB-231 or human prostate adenocarcinoma PC-3 cells) seeded onto the first monolayer. The second culture system consisted of conditioned medium collected from MDA-MB-231 or PC-3 cells, which was then added to the serum-free medium of cells representative of bone for 24 h.

Our results, shown in Figures 1 A-E, demonstrate that the presence of tumor cells causes the most dramatic results in endothelial cells, but an increase in MMP-1 protein is also observed in all other cells used. The addition of cancer cell-conditioned media only significantly increases MMP-1 protein in fibroblasts. In bone metastasis the presence of tumor cells can thus have an indirect stimulating effect on the expression of MMP-1, one of the major enzymes responsible for the initial degradation of native type I collagen, by cells resident in the bone. This might be of particular importance in the degradation of the organic bone matrix during the early stages of osteolysis, which has been shown to be both necessary and sufficient to activate osteoclastic bone resorption.

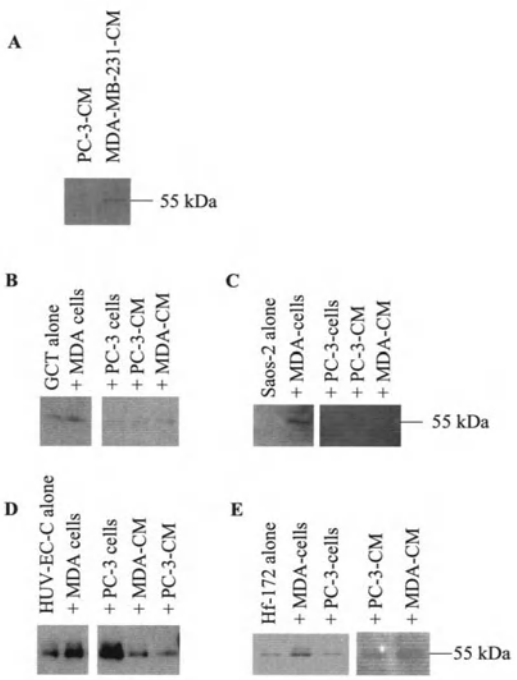

Figure 1. The effect of human bone-metastatic tumor cells on the interstitial collagenase MMP-1 expression in human cells representing stromal components of the bone (monocyte osteoclast-like GCT cells, osteosarcoma osteoblast-like Saos-2 cells, endothelial umbilical vein HUV-EC-C cells, and fibroblast Hf-172 cells). Subconfluent human prostate adenocarcinoma PC-3 and human breast adenocarcinoma MDA-MB-231 cells were incubated in serum-free media for 24 h. The conditioned media were collected and added to subconfluent GCT, Saos-2, HUV-EC-C, or Hf-172 cells for 24 h to constitute up to 50% of the media. Subconfluent GCT, Saos-2, HUV-EC-C, or Hf-172 cells were also co-cultured with MDA-MB-231 and PC-3 cells and their conditioned media were collected over a 24 h-period. Cell number-standardized samples were subjected to 10% SDS-PAGE and subsequent Western blotting to determine protein expression. We used a monoclonal MMP-1 antibody (0.5 □g/ml, Oncogene Research Products, Cambridge, MA) and subsequently a goat anti-mouse antibody conjugated with horseradish peroxidase (HRP) (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA). ECL chemiluminescence detection (Amersham, Canada) was used to visualize the proteins. (A) Western blot analysis for MMP-1 protein. MMP-1 (at 55 kDa) is expressed in 24 h-conditioned media from human bone metastatic breast adenocarcinoma MDA-MB-231 cells, but not from prostate adenocarcinoma PC-3 cells. Lanes 1 in (B), (C), (D), and (E) demonstrate MMP-1 expression in 24 h-conditioned media of the cells representing stromal components of the bone: (B) osteoclast-like GCT cells, (C) osteoblast-like Saos-2 cells, (D) endothelial HUV-EC-C cells, and (E) fibroblast Hf-172 cells. Lanes 2 and 3 show the results of the co-culture (24 h) experiments of PC-3 or MDA-MB-231 cells together with cells representing stromal components of the bone. Protein expression in cells representing stromal components of the bone 24 h after the addition of conditioned media derived from cancer cells (50%) is presented in lanes 4 and 5.

These results support the role of a membrane-bound, inducing factor on the tumor cells, such as EMMPRIN, since the most dramatic increases are observed when cells are co-cultured. However, the expression of EMMPRIN is probably not the only factor affecting the expression of MMPs, since both MDA-MB-231 and PC-3 cells express EMMPRIN (data not shown), but their pattern of induction of MMP expression is different. The observed differences could also be related to the differences in bone metastasis from breast and prostate cancer.

4. MATRIX METALLOPROTEINASE INHIBITORS

During the last decade synthetic MMP inhibitors have been developed for the treatment of cancer. They are able to effectively reduce or prevent the formation of metastases in a number of animal studies. Many MMP inhibitors, including batimastat, marimastat, AG3340 (Prinomastat), and BMS-275291 have been tested in clinical trials for various types of cancer. Unfortunately, most trials were unsuccessful and some were ended prematurely.

Batimastat (BB-94) was the first MMP inhibitor to be tested in cancer patients. It is a peptide-like analogue of the collagen substrate and a broad spectrum MMP inhibitor. *In vitro* studies show that batimastat is able to inhibit basement membrane degradation by human breast cancer cells (172), invasion of various cells into a reconstituted basement membrane (173) and degradation of type IV collagen (174). Batimastat has been shown to reduce the tumor burden of orthotopic xenografts of ovarian, colon, and breast cancer and to inhibit the formation of distant metastases in these murine models (149, 175-177). In a rat mammary carcinoma model batimastat was administered two days prior to surgical removal of primary tumors and continued for 73 days. At 120 days, 100% of the animals were disease-free compared to death in all controls. These results point to a potential for MMP inhibitors as a neoadjuvant or protective mode immediately prior to breast cancer surgery (178). Moreover, batimastat potentiates the activity of cisplatin, the most effective chemotherapy regimen for ovarian cancer patients, in an ovarian carcinoma mouse model (179). Phase I studies in patients with malignant ascites (180) and malignant pleural effusions (181) have been completed for this drug. Batimastat is well tolerated at doses that result in sustained plasma concentrations that are higher than the IC_{50}. Even though the phase I trials showed promising results, batimastat has been discontinued due to poor bioavailability.

Marimastat is a second-generation MMP inhibitor with better oral bioavailability. A meta-analysis using data from dose-finding studies for

advanced and serologically progressive cancers on a total of 415 patients shows that there is a significant effect of marimastat on the levels of cancer specific antigens (182). A pilot study of marimastat in advanced gastric cancer patients revealed that marimastat increases the fibrous tissue covering the tumors and decreases the haemorrhagic appearance at day 28 after start of the treatment (183). Phase I studies have also shown that marimastat in combination with chemotherapy agents such as doxorubicin, cyclophosphamide (184), 5-fluoruracil (185) or carboplatin (186) is well tolerated and the combination does not cause additional toxicity. A randomized, double-blind, placebo-controlled study using marimastat in non-resectable gastric cancer demonstrates an increase in both overall and progression-free survival (187).

Other MMP inhibitors, such as BAY 12-9566 and AG3340, reached the stage of phase III clinical trials (188), but were eventually halted. In animals, BAY 12-9566 has shown efficacy in murine models of B16/F10 melanoma and Lewis lung carcinoma (189) and in orthotopic tumor models of human breast cancer (190) and colon carcinoma (191). In combination with doxorubicin, plasma levels of BAY 12-9566 remain the same, however the haematological toxicity seems slightly more pronounced, but is not dose-limiting (192). In 2000, all clinical trials in cancer involving BAY 12-9566 were halted following a recommendation from an independent Data Safety Monitoring Board to close the non-small cell lung cancer trial due to excessive deaths in the BAY 12-9566 arm. AG3340 has been extensively tested in animal studies including the murine B16/F10 melanoma and Lewis lung carcinoma models (193), human malignant glioma (194), and colon adenocarcinoma models (195) and shows a high efficacy in these models, ranging from reduction in primary tumor size, invasion and number of metastases. A combination of AG3340 with carboplatin markedly increases survival of rats bearing human lung cancer xenografts (196). In addition to MMP inhibitory activity, AG3340 also has the potential to inhibit proliferation of human colon adenocarcinoma cells *in vivo* (194, 195) and induce apoptosis in human prostate adenocarcinoma PC-3 cells grown subcutaneously in nude mice (197). After prolonged treatment of animals with AG3340 the tumor-suppressing effect observed initially disappears, however, this does not seem to be due to induced resistance (198-200). Lack of effectiveness of AG3340 was eventually the reason to stop phase III clinical trials in advanced non-small cell lung cancer and prostate cancer.

One MMP inhibitor, BMS-275291, is still under investigation in a phase II trial involving hormone-refractory prostate cancer patients. BMS-275291 is an orally available broad-spectrum MMP inhibitor effective against MMP-1, MMP-2, MMP-7, MMP-9 and MT1-MMP in nanomolar concentrations.

BMS-275291 is able to dose-dependently inhibit lung metastases in mice after intravenous injection of B16/BL6 cells (201).

Furthermore, tetracyclines are potent inhibitors of cancer cell proliferation (202, 209-211), suggesting a direct indication for anti-tumor therapy. We have demonstrated that doxycycline is capable of inhibiting tumor cell growth in a concentration-dependent manner in bone metastasizing cancer cells (Figure 2B). Doxycycline is also able to inhibit MMP-2 and MMP-9 activity by 80% (Figure 2A) (162).

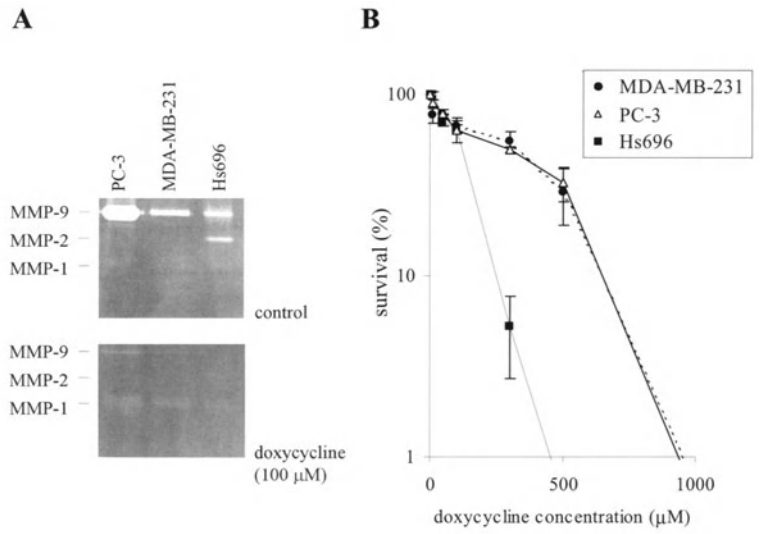

Figure 2. (A) Doxycycline-induced inhibition of MMP activity secreted by bone metastasizing cancer cells. Subconfluent human prostate adenocarcinoma PC-3, human breast adenocarcinoma MDA-MB-231, and Hs696 cells (human adenocarcinoma of unknown primary origin) were incubated in serum-free media for 24 h. The conditioned media were collected, concentrated and samples were subjected to gelatin enzymography to assess MMP-activity. The zymograms were incubated in substrate buffer in the absence (control) or presence of 100 μM doxycycline. Matrix metalloproteinase activity of 92-kDa type IV collagenase (MMP-9) and 72-kDa type IV collagenase (MMP-2) secreted by bone-metastasizing cancer cells was largely abolished by incubation in 100 μM doxycycline. (B) Survival curves of bone metastasizing human cancer cells (PC-3, MDA-MB-231, and Hs696) as measured by colony forming assay in a 24 h-treatment protocol. Values are represented as ratios of treated over corresponding control. Each value represents mean data (± S.E.) of three experiments carried out in triplicate. Mean survival for human prostate adenocarcinoma PC-3 cells treated with 500 μM doxycycline was 0.6±1.0%, for human breast adenocarcinoma MDA-MB-231 and human adenocarcinoma Hs696 cells treated with 1000 μM doxycycline the mean survival amounted to 0.7±0.5 and 0.6±0.0%, respectively. Mean values reached significance ($p<0.05$) for PC-3 cells at 50 μM doxycycline, for MDA-MB-231 and Hs696 cells at 100 μM doxycycline.

5. THE FUTURE OF BONE METASTASIS TREATMENT

A targetted therapy consisting of a combination of bisphophonates, MMP inhibitors, and/or tetracyclines could potentially be more successful. MMP inhibitors that might prove to be useful in cancers with an overexpression of MMP-2 or MMP-9 might actually not be the best approach for therapy of bone metastasis. When the tumors spread to the bone the inhibition of MMPs, such as the interstitial collagenases MMP-1, MMP-8, and MMP-13, might be more beneficial in the reduction or prevention of bone destruction. MMP inhibitors are currently being designed for use in rheumatoid arthritis to prevent bone and cartilage loss. They are more effective against the interstitial collagenases, the same MMPs that are also involved in the breakdown of cartilage components. A combinatory and/or sequential treatment with MMP inhibitors directed towards the interstitial collagenases, such as bisphosphonates, and/or tetracyclines might be beneficial. Further investigation into the potential of combination treatment might be a valuable approach towards the ultimate goal of controlling bone metastasis.

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LIST OF ABBREVIATIONS:

MMP: Matrix Metalloproteinase
TIMP: Tissue Inhibitor of Matrix Mettalloproteinase
EMMPRIN: Extracellular Matrix Metalloproteinase Inducer

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Chapter 13

THE ROLE OF BISPHOSPHONATES IN BONE METASTASIS

Mary MacKenzie and Pierre Major
Juravinski Cancer Centre, Hamilton, Ontario, Canada

1. INTRODUCTION

Bisphosphonates are analogs of pyrophosphate, which is a naturally occurring regulator of bone mineralization with the chemical structure P-O-P (see Figure 1). These compounds were first synthesized in Germany in 1865. Their initial use was industrial (textile, fertilizer and oil industries) and for prevention of mineral scaling (due to their ability to inhibit calcium carbonate precipitation). The biological properties of these compounds, however, were not explored until the 1960's (1, 2).

In the decades since their first clinical use, bisphosphonates have become an important tool in the treatment of a broad spectrum of diseases including Paget's disease, osteoporosis, and cancer. The role of bisphosphonates in the treatment of malignant disease is expanding. These agents have been successfully used in the treatment of bone metastases and malignancy-associated hypercalcemia; and their benefit in the adjuvant setting is being evaluated.

Bisphosphonates exert their effects through a variety of mechanisms. Their molecular mechanism of action, as well as their effects on osteoclasts, osteoblasts and tumor cells will be reviewed here.

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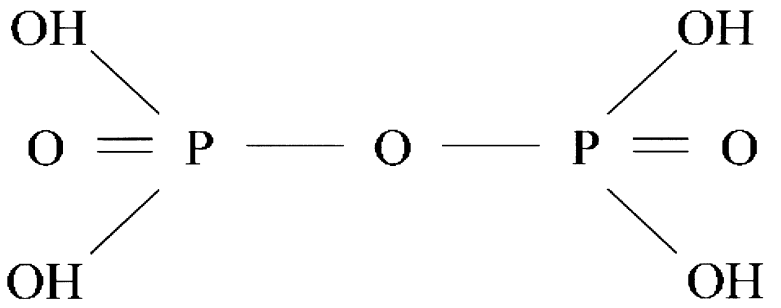

Figure 1. Pyrophosphate

2. MOLECULAR MECHANISMS OF ACTION

In bisphosphonates, carbon has replaced the central oxygen of the pyrophosphate structure. First generation bisphosphonates include clodronate and etidronate. Newer generation bisphosphonates (pamidronate, alendronate, ibandronate, risedronate and zoledronic acid) have a side chain containing one or more nitrogen atoms (see Figure 2). All bisphosphonates form a 3D structure capable of binding divalent ions including calcium. The affinity for calcium can be increased further if one of the side chains is a hydroxyl or a primary amino group (as with second generation bisphosphonates) because this allows the formation of a tridentate configuration that is able to bind to calcium more avidly (3).

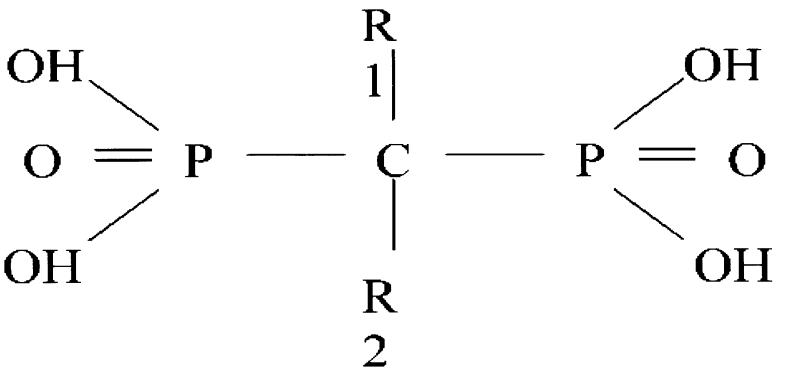

Figure2. Bisphosphonate

In both tumor cells and osteoclasts, first generation bisphophonates act through the formation of cytotoxic ATP-analogues after internalization by cells. These metabolites accumulate in the intracellular space and inhibit crucial ATP-dependant enzymes (4).

Newer, nitrogen-containing bisphosphonates exert their effect by inhibition of the mevalonate pathway (Figure 3). One enzyme that is specifically inhibited in this pathway is farnesyl diphosphate synthase (5). Inhibition of this enzyme results in a decrease in prenylation (post-translational modification) of small GTP-binding proteins that are essential for normal cell function. Such GTP-binding proteins include ras, rac and rho. Senaratne et al. (6) have shown that, in two lines of breast cancer cells, zoledronic acid impaired membrane localization of ras. This would indicate that prenylation (in this case, farnesylation) of this protein is reduced by the presence of this bisphosphonate. Likewise, Bergstrom et al. (7) also identified farnesyl diphosphate synthase as the selective target of alendronate in the mevalonate pathway.

This effect of nitrogen-containing bisphosphonates is not, however, limited to farnesylation. Coxon et al. (8) found that the inhibitory effect of nitrogen-containing bisphosphonates on bone resorption more likely results from the loss of a different set of prenylated proteins; the geranylgeranylated proteins. In their assays, GGTI-298 (a specific inhibitor of geranylgeranyl transferase I) prevented osteoclast formation in murine bone marrow cultures, inhibited bone resorption and promoted osteoclast apoptosis. In contrast to that finding, FTI-227 (a specific inhibitor of farnesyl transferase) had little effect on osteoclast apoptosis and did not inhibit bone resorption.

These results underscore the importance of geranylgeranylated proteins in osteoclast function.

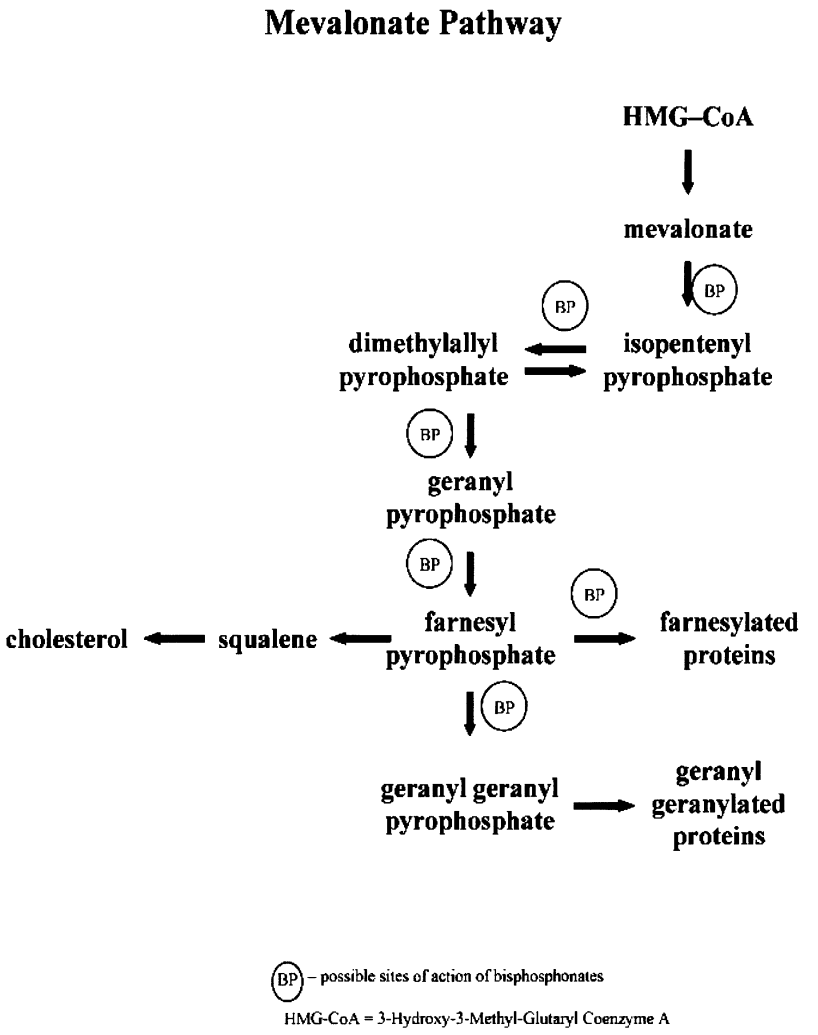

Figure 3. The Mevalonate Pathway of HMG metabolism generates prenylated proteins essential for cell signal transduction. Potential sites of action of bisphosphonates are indicated.

3. CELLULAR MECHANISMS OF ACTION

Bisphosphonates exert their effects on a broad array of cell types, including both skeletal and soft tissues (Figure 4). These agents do, however, have a very strong chemical affinity for calcium phosphate. It is this feature that is responsible for their particular localization to bone. Thus, the majority of their effects are exerted on the skeleton. These effects are detailed in the following sections.

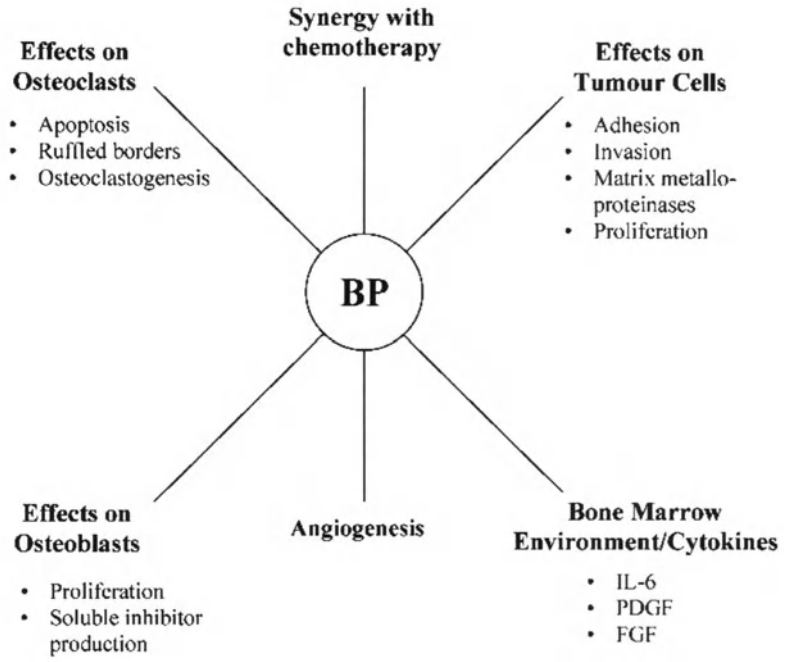

Figure 4. Bisphosphonates exert their effects on bone, the bone marrow microenvironment, tumour cells and microvasculature via the pathways indicated.

3.1 Effects on Osteoclasts

The potent effect of bisphosphonates on osteoclasts is explained in part by their high affinity for calcium phosphate and consequent localization to bone. Bisphosphonates present in the bone lacuna are internalized by osteoclasts into endocytic vacuoles. The mechanism by which

bisphosphonates subsequently cross the vacuolar membrane is not well understood.

3.1.1 Apoptosis of Osteoclasts

Both nitrogen and non-nitrogen-containing bisphosphonates result in osteoclast apoptosis. Hughes et al. (9) observed the effects of three bisphosphonates (risedronate, pamidronate and clodronate) on osteoclast survival. *In vitro* treatment of osteoclasts with bisphosphonates was again associated with 4- to 24-fold increase in the proportion of osteoclasts with apoptotic changes. This observation was repeated in normal mice, mice with increased bone resorption and mice with osteolytic metastases. Other authors have noted that in addition to these apoptotic changes, osteoclast actin-rings are disrupted (10).

Building on these observations, Ito et al. (11) observed the effects of a third generation bisphosphonate (incadronate) on the femur and tibia of rats. Apoptosis was identified in osteoclasts on the basis of characteristic changes in cell and nuclear morphology, including pyknotic nuclei with condensation and margination of heterochromatins and DNA fragmentation. These investigators also noted enlargement and fusion of nuclear envelopes, leading to leakage of nuclear contents into the cytoplasm in the late stages of apoptosis. Degradation of cellular organelles, including the Golgi apparatus, was seen.

This process of bisphosphonate-induced osteoclast apoptosis entails the activation of caspases. Caspases are a group of cysteine proteases that cleaves intracellular substrates including poly-adenosine diphosphate, protein kinase C, and nuclear lamin proteins. The major osteoclast protease activated by bisphosphonate treatment is caspase-3-like protease. This protease, which specifically cleaves peptides with the amino acid sequence asp-glu-val-asp, thus plays an important role in osteoclast apoptosis (4).

Selander et al. (12) studied the effects of clodronate and pamidronate on both osteoclasts and macrophages (which are ontogenetically related to osteoclasts). The investigators assessed the effects of several inhibitors of the apoptotic cascade in preventing clodronate-induced programmed cell death. None of the inhibitors tested could completely prevent clondronate-induced apoptosis. Partial protection was seen with staurosporine and homocysteine, suggesting that cytoplasmic protein kinase C-activated mechanisms play a role in clodronate-induced osteoclast apoptosis.

Other investigators have raised the question of whether osteoclast apoptosis is an absolutely necessary step in the process of bisphosphonate-induced inhibition of bone resorption. Halasy-Nagy et al. (13) studied the effects of both nitrogen-containing (alendronate and risedronate) and non-

nitrogen-containing (clodronate and etidronate) bisphosphonates on osteoclasts *in vitro*. The investigators showed that the anti-apoptotic cascade inhibitor Z-VAD-FNK did not prevent inhibition of resorption of bone seen with nitrogen-containing bisphosphonates. In contrast to this finding, Z-VAD-FMK did block clodronate- and etidronate-mediated inhibition of bone resorption. This data suggests that induction of apoptosis plays a major role in etidronate and clodronate-mediated inhibition of resorption, but alendronate and risedronate suppression of resorption is independent of their effects on apoptosis.

Other experimental findings have also supported this assertion that osteoclast apoptosis is not always required in order to see inhibition of bone resorption. These studies show that inhibition of bone resorption by nitrogen-containing bisphosphonates was not associated with a decrease in osteoclast numbers except at very high concentrations. Hence, as well as causing osteoclast cell death, bisphosphonates can cause more subtle changes in osteoclast function that affect their ability to resorb bone (3).

3.1.2 Osteoclast Ruffled Borders

After osteoclasts adhere to bone surface, they exhibit features of highly polarized cytoplasmic organization, including ruffled borders (this border is the tortuous area of osteoclast plasma membrane that lies adjacent to the bone surface, and is essential for bone resorption) and clear zones (14). The clear zone surrounds the ruffled border and seals the extra-cellular resorbing compartment in which bone resorption takes place. Osteoclasts adhere to the bone surfaced though structures in the clear zones called podosomes. These podosomes consist of dots containing F-actin.

One consistent feature of osteoclasts treated with bisphosphonates (*in vitro* or *in vivo*) is the lack of this ruffled border. Early on in the clinical development of bisphosphonates, Miller and Jee (15) studied the electron microscopic features of the bones of growing rats following administration of the bisphosphonate C12MDP. The ruffled borders were generally less extensive than in control rats. Similarly, Sato et al. (16) demonstrated that osteoclasts from adult rats, when treated with alendronate, lost their ruffled border but the clear zone remained intact.

The effects of tiludronate on osteoclast morphology have been studied in mouse culture systems (14). The results of this experiment showed that tiludronate is selectively incorporated into polarized osteoclasts that have a ruffled border (i.e., those osteoclasts that are functioning). Similarly, Ito et al. (11) examined the cytochemical and ultrastructural features of osteoclasts after treatment with a third generation bisphosphonate. These osteoclasts became devoid of ruffled borders and detached from bone surface.

The same authours (17) also examined the effects of 3rd generation bisphosphonate on osteoclasts in osteosclerotic mice (*oc/oc* mice). In such mice, osteoclasts are small and devoid of ruffled borders. Therefore, bone resorption is reduced. In these mice, after treatment with bisphosphonates, the osteoclasts detached from the bone surface and became apoptotic. This evidence indicates that osteoclasts that lack ruffled borders are nevertheless still affected by bisphosphonates. Therefore, these cells must absorb the bisphosphonates from a site other than the ruffled borders.

Finally, Alakangas et al. (18) treated rat osteoclasts with alendronate. In this experiment, widening of sealing zone areas, incomplete organization of tight attachments and ruffled borders were observed. Osteoclasts appeared partially detached from bone surface (by contrast, clodronate bone surfaces were thoroughly resorbed). The authors concluded that, following treatment with alendronate, osteoclasts are inactivated by mechanisms that impair vesicle transport, and apoptosis is only a secondary phenomenon.

3.1.3 Effects on Osteoclastogenesis

Osteoclasts derive from precursor cells of macrophagic/monocyte lineage. These cells express receptor activator of NF-kappaB (RANK), which is a member of the tumour necrosis factor receptor superfamily. Following activation of RANK by its ligand, RANKL, differentiation, proliferation and survival of proteoclasts is promoted. RANKL is largely produced by cells of osteoblast lineage. It can also be secreted by immune cells and by some tumour cells. RANKL is, physiologically, kept in check by osteoprotegerin (OPG). OPG serves as a soluble decoy receptor, which blocks RANKL and thus prevents bone resorption. This normal balance between RANKL and OPG regulates the physiologic process of bone resorption and remodelling. When, however, this balance is disrupted by malignant tumours, skeletal complications of cancer ensue (19).

The effects of bisphosphonates on osteoclastogenesis in fetal rat calvariae have been studied by Evans et al. (20). All bisphosphonates tested were able to inhibit osteoclast formation at low concentrations. Little or no effect on osteoblasts was seen. Evidence also indicates that, in order for bisphosphonates to exert their anti-osteoclastogenic effects, they must be bound to bone mineral (21).

In an effort to elucidate the mechanism of this inhibition of osteoclastogenesis, Nishikawa et al. (22) examined the effects of the bisphosphonate YM175 on osteoclast-like multinucleated cell formation in cell culture. When osteoclast precursors of different tissue origins were treated with YM175, and co-cultured with calvarial osteoblasts, inhibition of osteoclast-like multinucleated cells was observed. When these osteoclast

precursors were co-cultured with supporting cells of different origins (i.e. not osteoblasts), the results of treatment with YM175 varied. These findings suggest that YM175 inhibits osteoclastogenesis through its action on supporting cells of osteoblast lineage, rather than acting directly on the osteoclast precursors.

Clohisy et al. (23) used osteopetrotic (osteoclast-deficient) mice as an animal model to study the effect of pamidronate on a sarcoma that induces osteoclastogenesis. Experiments with this animal model suggested that pamidronate decreases the number of osteoclasts through its effects on myeloid precursor cells.

A separate mechanism of osteoclastogenesis inhibition is through increased production of osteoprotegerin from osteoblasts. A recent study confirmed that both pamidronate and zoledronic acid result in increased osteoprotegerin mRNA levels and OPG secretion in human osteoblasts (24).

In contrast to the above evidence, some investigators have found that bisphosphonates do not affect osteoclast formation. Breuil et al. (25) found that formation of osteoclast-like multinucleated cells from peripheral blood mononuclear cells was not inhibited by alendronate. Despite this data, the majority of studies support the hypothesis that some of the anti-resorptive activity of bisphosphonates is due to inhibition of osteoclastogenesis.

3.2 Effects on Osteoblasts

Reinholz et al. (26) studied the direct effects of bisphosphonates on cell proliferation, gene expression and bone formation in cultured fetal osteoblasts. Osteoblast cell proliferation was decreased, and cytodifferentiation was increased after treatment with pamidronate. These same authors, in a later study (27), found that zoledronic acid also induces human osteoblast differentiation via inhibition of the mevalonate pathway (i.e., the same pathway that is inhibited in osteoclasts). These *in vitro* observations, however, are probably not of physiologic significance, as is it known that bisphosphonates do not inhibit bone formation on a clinically important level (3).

Vitte et al. (28) studied the effect of ibandronate and alendronate on an osteoblastic cell line. When osteoclasts were cultured in medium that was taken from bisphosphonate-treated osteoblasts, pit formation (a measure of resorptive activity) was reduced by 50%. This suggests that the osteoblasts, when treated with bisphosphonates, secrete a soluble factor that inhibits osteoclast activity (later identified as osteoprotegerin). This hypothesis is supported by similar experiments by Yu et al. (29).

Alendronate has also been found to disrupt the modulatory effect of 1,25-dihydroxyvitamin D3 (a physiologic regulator of bone resorption) on

parathyroid hormone-related peptide (PTHrP) production in human osteoblast cells. PHTrP is produced by several tumour types, and acts on PTH receptors to cause increased bone resorption. An increase in calcium influx appears to be involved in the mechanism mediating this effect of alendronate (30).

4. EFFECTS ON TUMOR CELLS

Many patients with malignant disease will eventually develop bone metastases. The incidence of bone involvement varies with the tumor type; in some diseases (multiple myeloma, breast and prostate cancer) the probability of bone metastases occurring is in excess of 65%. Bisphosphonates, through their effects on tumor adhesion, invasion, proliferation and on angiogenesis (in addition to their effects on bone), may have the potential to influence the clinical course of skeletal metastases.

4.1 Tumor Adhesion to Bone

Bisphosphonates strongly inhibit the binding of many human tumor cell line types, including breast and prostate, to bone. This effect has been seen regardless of whether the bone or the tumor cells were treated. Clezardin et al. (31) found that nitrogen-containing bisphosphonates inhibit tumor cell adhesion in a dose-dependant manner. The mechanism by which nitrogen-containing bisphosphonates inhibit tumor cell adhesion remains poorly understood. It is likely that modulation of cell adhesion molecules including cadherin, laminin and the integrins is involved.

Tumor adhesion to bone is a crucial step in the metastatic process. van der Pluijm et al. (32) used two *in vitro* models of bone matrix (cortical bone slices and cryostat sections of trabecular bone from neonatal mouse tails) to study the effects of bisphosphonate treatment on tumor adhesion. With these models, it was shown that four bisphosphonates (pamidronate, olpadronate, alendronate and ibandronate) inhibited adhesion and spreading of breast cancer cells in a dose-dependant manner.

The effect of bisphosphonates on breast and prostate carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices has been studied (33). Bisphosphonate pre-treatment of tumor cells inhibited tumor cell adhesion to unmineralized and mineralized osteoblastic extracellular membranes in a dose-dependant manner. They did not, in contrast, affect adhesion of normal cells (fibroblasts) to extracellular membranes.

4.2 Tumor Cell Invasion/ Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a group of zinc-dependant endopeptidases involved in tissue remodeling (34). They are crucial to the ability of tumor cells to invade and metastasize, and are important in the maintenance and growth of metastatic foci. Nitrogen-containing bisphosphonates inhibit several MMPs. This inhibition is reversible with the addition of zinc, suggesting that the ability of bisphosphonates to chelate divalent ions is the mechanism by which they inhibit MMPs.

Teronen et al. (35) confirmed that the function of various MMP's is inhibited *in vitro* by bisphosphonates. In enzyme activity tests using purified and recombinant enzymes, they observed that the inhibition of MMP-1,2,3,7,8,9,12,13,14 activity by clodronate, alendronate, zoledronic acid, and nedrinate. Clodronate downregulates the expression of MT1-MMP protein and mRNA in several cell lines. Also, several bisphosphonates decreased the invasion of melanoma and fibrosarcoma cell lines through the artificial basement membrane, Matrigel.

Clodronate also inhibits MMP-8-mediated degradation of casein and type 1 collagen, and has inhibitory effects on MMP-9 and –2. The nitrogen-containing bisphosphonates pamidronate and alendronate inhibits activity of MMP-3. Zoledronic acid has been shown to inhibit MMP-3.

Derenne et al. (36) found that zoledronic acid inhibits the production of MMP-1 (the major MMP involved in the initiation of bone metastases) by bone marrow stromal cells. Similarly, Ichinose et al. (37) investigated the role of bisphosphonates in the production of MMP-2 from human osteoblasts. Production was reduced in the presence of physiologic levels of plasmin.

However, conflicting data prevents the drawing of firm conclusions. For example zoledronic acid did not cause decreased expression of MMP-2 and -9 from breast cancer cells (33).

4.3 Tumor Cell Proliferation and Survival

An inhibitory effect of bisphosphonates on the growth of myeloma, melanoma, breast and prostate cancer cell lines has been observed (31). Concentrations of 10^{-5} to 10^{-4} are required to inhibit cellular proliferation. These concentrations are significantly higher than would be present in serum. However, they may be reached in bone at sites of active resorption, given that bisphosphonates avidly adhere to bone. As is true for osteoclasts, the major mechanism responsible for tumor cell apoptosis appears to be caspase activation (38).

The direct effects of bisphosphonates on breast cancer cells have been reviewed by Senaratne et al. (6). This author has also investigated the *in vitro* effects of zoledronic acid, pamidronate, and clodronate on growth, viability, and apoptosis in three human breast cancer cell lines. In these experiments, all three bisphosphonates caused a significant reduction in cell viability. The most potent of these was zoledronate. Treatment was also accompanied by fragmentation of DNA (a feature of apoptosis). This increase in apoptotic rate was associated with decreased expression of the anti-apoptotic protein Bcl-2.

The inhibitory effects of zoledronic acid on bone metastases of prostate cancer have recently been examined (39). Treatment with zoledronate decreased proliferation of prostate cancer cells *in vitro*, causing G1 arrest and apoptosis. This finding was not, however, repeated *in vivo* using a murine model with subcutaneous xenografts of PC-3 and LuCaP 23.1 cells (no reduction in tumor volume was seen). The growth of osteoblastic and osteolytic metastases of prostate cancer in this experiment was inhibited significantly.

4.4 Effects on Bone Marrow Environment/Growth Factors/Cytokines

Numerous growth factors are present in the bone marrow microenvironment; these include platelet-derived growth factor (PDGF), fibroblast-growth factor (FGF), and transforming growth factor beta (TGF-β). These factors are release during bone resorption and may stimulate tumor cell proliferation. In turn, tumor cells often secrete PTHrP. This further stimulates osteoclasts to resorb bone, and thus a "vicious cycle" is established (Figure 5). Through inhibition of osteoclastic resorption, bisphosphonates can decrease the release of growth factors from bone.

As previously mentioned, Derenne et al. (36) investigated the effects of zoledronic acid on myeloma cell growth. In addition to inhibiting the production of MMP-1, zoledronic acid induced a significant inhibition of the constitutive production of interleukin-6 by bone marrow stromal cells. However, in a separate experiment, alendronate and etidronate had no effect on the synthesis of IL-6 or IL-11 in normal human osteoblasts in culture. This conflicting evidence suggests that osteoclast-mediated effect on bone resorption may not be exerted through these cytokines.

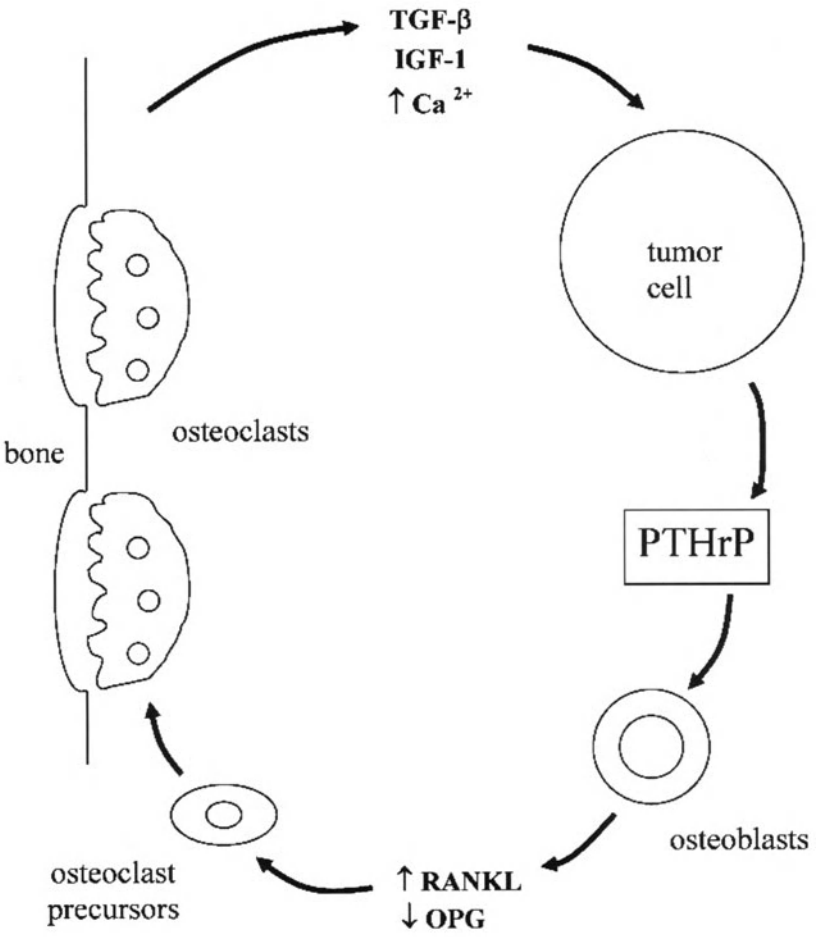

Figure 5. The "vicious cycle" between cancer cells and the bone microenvironment. Breakdown of bone by osteoclasts promotes the release of mitogenic factors including TGF-β and IGF-1. TGF-β then promotes the release of PTHrP by tumour cells, thus leading to further bone breakdown

TGF- β = Transforming Growth Factor - β
IGF-1 = Insulin-like Growth Factor 1
PTHrP = Parathyroid Hormone Related Peptide
RANKL = Receptor Activator of NF-Kappa B ligand
OPG = Osteoprotegerin

4.5 Effects on Angiogenesis

Angiogenesis (the formation of new, tumour-associated, blood vessels) is essential for the establishment and growth of primary tumors and metastases. There is abundant evidence that bisphosphonates inhibit this process.

Zoledronic acid has been proven *in vitro* to inhibit the proliferation of human umbilical vein endothelial cells (an assay of antiangiogenic activity) normally induced by basic fibroblast growth factor, vascular endothelial growth factor and serum. When administered systemically to mice, zoledronic acid also potently inhibits the angiogenesis induced by subcutaneous implants impregnated with basic fibroblast growth factor (40).

A tissue model of angiogenesis has been developed using the prostates of castrated rats, in which the effects of bisphosphonates on testosterone-induced revascularization could be studied. Ibandronate and zoledronate, when given in combination with testosterone, induced a 50% reduction of revascularization in tests of blood vessel immunostaining (41).

Croucher et al. (42) investigated the effect of zoledronic acid on the development of bone disease, tumor burden and disease free survival on the 5T2MM model of multiple myeloma. Myeloma cells were injected intravenously into mice. The animals were then treated with zoledronic acid. Treatment was associated with a reduction in tumor burden and reduced angiogenesis as measured by microvessel density in myelomatous bone lesions.

Zoledronic acid modulates the endothelial cell surface receptors involved in angiogenesis. Such receptors, including alpha vBeta3 and alpha vBeta5 integrins and the 65kD laminin receptor, were downregulated on human endothelial cells after treatment with zoledronic acid (38).

In terms of clinical evidence for inhibition of angiogenesis, Santini et al. (43) evaluated the modification of angiogenetic cytokines after pamidronate infusion in 25 cancer patients with bone metastases. Basic Vascular Endothelial Growth Factor (which has been cited as a possible surrogate marker for angiogenic activity) levels decreased significantly at 1, 2 and 7 days after pamidronate treatment.

4.6 Synergism with Chemotherapy

As the use of bisphosphonates in the treatment of bone metastases increases, more patients will be receiving such treatment in combination with palliative chemotherapy. The evidence relating to the possible synergistic effects of bisphosphonates and chemotherapy is discussed below.

The anti-tumor effects of several chemotherapeutic agents are potentiated by bisphosphonates. Clezardin et al. (31) has studied the effects of combined

ibandronate and taxanes in breast cancer cell lines. Inhibitory effects on adhesion and invasion of bone were observed. Also, zoledronic acid combined with paclitaxel exerts a synergistic proapoptotic effect on breast cancer cell lines (44). In murine models of bone metastases from breast carcinoma cells, the combination of ibandronate and doxorubicin suppressed bone (and visceral) metastases more effectively than either agent alone (45).

Zoledronic acid has also shown an additive effect when combined with the COX-2 inhibitor SC236. Witters et al. (46) observed that zoledronic acid alone caused a 23% reduction in growth of prostate cancer cells, while SC236 caused a 40% reduction. When these agents were combined, a 60% reduction in tumor growth was observed.

5. CLINICAL USE OF BISPHOSPHONATES IN CANCER PATIENTS

Skeletal metastases are a common problem, particularly with cancer of the breast, prostate, lung and with multiple myeloma. Complications of skeletal metastases include bone pain, pathologic fractures, hypercalcemia and spinal cord compression. Several treatment modalities are available for the treatment of bone pain, including radiotherapy, analgesics and bisphosphonates. Prior to the era of bisphosphonates, hypercalcemia was treated acutely with volume expansion, calcitonin, plicamycin, and loop diuretics. These agents were of limited efficacy, and today, bisphosphonates are considered to be standard management in the care of such patients.

5.1 Breast Cancer

At least 25% of women diagnosed with breast cancer will eventually develop bone metastases. On average, these patients will have a complication (such as pain and fracture) from their bone metastases once every three to four months (47).

Several large multicentre trials have examined the effects of bisphosphonates in the treatment of both early and advanced breast cancer (see Table 1). A systematic review of randomized trials has been performed (48). In eight studies that included 1962 women with advanced breast cancer and existing bone metastases, bisphosphonates reduced the risk of developing a skeletal event by 14% (relative risk 0.86, 95% confidence interval 0.8-0.91;$p<0.00001$). All studies of intravenous pamidronate and oral clodronate in women with advanced breast cancer and bone metastases showed significant delays in median time to a skeletal event.

Bisphosphonates are also associated with a reduction in bone pain; compared with placebo or no treatment, bisphosphonates significantly improved pain scores in four trials. Treatment with bisphosphonates does not; however, appear to affect survival in women with advanced breast cancer.

Table 1. Clinical Trials of Bisphosphonates in Breast Cancer Patients

Trial	Compound /Dose	Patients	Endpoints	Outcome	Comments
Paterson et al., 1993[55]	Clodronate 1600 mg/day	173	HCM, radiotherapy, fractures	Fewer vertebral fractures and less HCM, no difference in complications	104 patients died prior to 18 month follow-up
Kanis et al., 1996[56]	Clodronate 1600 mg/day	133	Development of bone mets and related clinical events	Fewer bone mets and clinical events; no difference in proportion of affected patients	Prevention study
Hortobagyi et al., 1996[57]	Pamidronate 90 mg IV vs. placebo	382	Median time to SRE; proportion of patients with SRE	Time to SRE:1.31m vs. 7 m (favor pamidronate);43 % with SRE for pamidronate. vs. 56%	
Diel et al., 1998[49]	Clodronate 1600 mg/day	302	Incidence and extent of new metastases	Lower incidence of osseous and visceral mets in the treatment group	Prevention study in patients with tumor cells in bone marrow
Kristensen et al., 1999[58]	Clodronate 1600 mg/day	100	Skeletal events including HCM, fractures and radiotherapy	Temporary reduction in number of skeletal events in treatment group	No effect on disease progression in bone or survival
Theriault et al., 1999[59]	Pamidronate 90 mg IV vs. placebo	372	Median time to SRE; proportion of patients with SRE	10.4 vs. 6.9 mos. (favor pamidronate); 56% pamidronate with SRE compared to 67% placebo	treatment X 28 weeks; more vomiting leukopenia with pamidronate than placebo
Saarto et al., 2001[50]	Clodronate 1600 mg/day daily vs. control	299	Incidence bone mets, nonosseous mets, OS, DFS	No difference in bone mets (21% vs. 17%); more nonosseous mets in clodronate	Prevention trial, patients received adjuvant chemotherapy and hormonal

Trial	Compound /Dose	Patients	Endpoints	Outcome	Comments
				group (43% vs, 25%); OS and DFS lower in clodronate group	therapy
Rosen et al., 2001[60]	Zoledronic acid 4 mg vs. 8 mg IV vs. Pamidronate 90 mg IV	1648	SRE	No difference in proportion with SRE's on zoledronic acid decreased incidence of bone radiation	Patients with skeletal mets from breast cancer or osteolytic myeloma
Powles et al., 2002[61]	Clodronate 1600 mg/day vs. placebo	1069	Relapse in bone and other sites, mortality, toxicity	Sig. reduction in bone mets during treatment period only; no difference in visceral mets. Sig. reduction in mortality	Prevention trial

HCM: hypercalcemia
SRE: Skeletal Related Events
OS: Overall survival
DFS: Disease free survival

The data regarding the use of bisphosphonates in early breast cancer is less compelling. In three studies of oral clodronate that included 1680 women with early breast cancer, there was borderline evidence of a reduction in the risk of developing skeletal metastases (RR 0.73, 95% CI 0.55-0.98;p=0.04). It should be noted that the populations of these trials were quite heterogeneous. Diel's trial of oral clodronate in 302 early breast cancer patients who were felt to be at high risk for recurrence (on the basis of bone marrow micrometastases) showed a lower incidence of bone metastases, and surprisingly, a lower incidence of visceral metastases in the clodronate group (49). This study was, however, refuted by Saarto's trial of 299 patients with node-positive operable breast cancer; here, bone metastases were detected with equal frequency in both the treatment and control groups (50). Interestingly, there was a *higher* incidence of non-osseous metastases and *lower* overall survival in the treatment group.

5.2 Multiple Myeloma

Multiple myeloma is a neoplastic disorder of plasma cells, and accounts for 10% of all haematologic malignancies. The mechanism of bone

destruction in myeloma is a result of both increased osteoclast and decreased osteoblast activity. These bone changes frequently lead to lytic lesions, particularly within the vertebral column. Hypercalcemia also will develop in 25% of patients with myeloma (51). For these reasons, bisphosphonates are increasingly being used in the management of this disease (see Table 2).

Table 2. Clinical Trials of Bisphosphonates in Multiple Myeloma Patients

Trial	Compound/ Dose	Patients	Endpoints	Outcome	Comments
Belch et al., 1991[62]	Etidronate 5/mg/kg/day	173	Height, HCM, path fractures, bone pain	No difference from placebo for any measure	Higher dose of 20mg/kg/day discontinued
Lahtinen et al., 1992[63]	Clodronate 2400mg/day	336	Osteolytic progression	Less progression with clodronate	No effect on fractures, bone pain or HCM
Brincker et al., 1998[64]	oral pamidronate 300 mg/day	300	Skeletal complications	No difference from placebo for skeletal-related morbidity	No influence on survival or hypercalcemia. Less pain in treatment group
Berenson et al., 1998[65]	Pamidronate 90 mg intravenously	392	SRE	Trend to lower SRE rate in treatment group (p=0.15)	No survival benefit
Terpos et al., 2000[66]	Pamidronate 90 mg intravenously	62	SRE	No changes in SRE's	Given synchronously with chemotherapy, pamidronate reduces markers of bone turnover
McCloskey et al., 2001[67]	Clodronate 1600 mg/day	619	Pathologic fracture, HCM, performance status, bone pain	Fewer fractures, no difference in HCM; survival benefit only in subgroup with no skeletal fractures at presentation	Follow up > 8 years
Rosen et al., 2001[60]	Zoledronic acid 4mg vs. 8 mg IV vs. Pamidronate 90mg iv	1648	SRE	No difference in proportion with SRE's on zoledronic acid decreased incidence of bone radiation	Patients with skeletal mets from breast cancer or osteolytic myeloma
Menssen et al., 2002[68]	intravenous ibandronate 2mg iv vs placebo		SRE	No difference in SRE's bone pain, median survival	Post hoc analyses showed suppression of bone turnover markers

A systematic review of trials related to the use of bisphosphonates in myeloma has been conducted (52). Eleven trials including 1113 patients were analysed. All were randomised trials comparing bisphosphonates to placebo or no treatment. The pooled analysis of these trials showed a beneficial effect of bisphosphonates in terms of the prevention of pathological vertebral fractures and improvement of pain (Odds Ratio 0.59; 95% CI 0.45-0.78; p=0.0001). There was no significant effect of bisphosphonates on mortality, reduction of non-vertebral fractures, or on the incidence of hypercalcemia.

5.3 Prostate Cancer

Please also see Table 3 for an overview of clinical trials of bisphosphonates in prostate cancer.

Excluding superficial skin cancers, prostate cancer is the most common malignancy afflicting North American men (51). This cancer has a strong propensity to metastasize to bone, often in the form of osteoblastic lesions. These metastases most commonly affect the pelvis, ribs, and spine. Those that affect the spine have the potential to cause postural changes, deformity, and, when they compress the spinal cord, significant neurologic impairment.

Several clinical trials have assessed the potential benefit of bisphosphonates in the management of prostate cancer patients with bone metastases. Many of the initial trials were statistically underpowered to detect any benefit in terms of pain or skeletal event rate. Most of these trials did not support the role of bisphosphonates in the treatment of prostate cancer-related bone pain.

A larger, more recent trial (53) has evaluated the role of zoledronic acid in prostate cancer patients. Two doses (8 and 4 mg) were compared to placebo. The 8mg arm was terminated prematurely due to concerns regarding renal toxicity. The trial showed a clinically significant advantage for zoledronic acid compared to placebo in terms of reduction of the proportion of patients any skeletal complication or pathologic fracture. Zoledronic acid also significantly reduced bone pain compared with placebo at 3 and 9 months.

Prostate cancer is often treated with androgen deprivation therapy, and this treatment has the potential to cause osteoporotic changes, thus compounding the deleterious bone effects of the disease itself. The role of clodronate in the prevention of osteopenia has been studied by Smith et al. (54) in patients with nonmetastatic prostate cancer who are receiving androgen deprivation therapy. Patients receiving clodronate had an increase in bone density of 5.6%. Those receiving placebo had a mean decrease in

bone density of 2.2%. Although this evidence is preliminary, it would support the use of clodronate in this setting.

Table 3. Clinical Trial of Bisphosphonates in Prostate Cancer Patients

Trial	Compound/ Dose	Patients	Endpoints	Outcome	Comments
Smith et al., 1989[69]	Etidronate 7.5 mg/kg IV for 3 days then 400 mg/day oral	57	Bone pain, skeletal lesions	No difference from placebo	Four treatment groups
Strang et al., 1997[70]	Clodronate intravenously followed by orally 1600mg/day vs. placebo	55	Pain assessment with visual analog scale	No difference in pain	Trial ended prematurely due to poor recruitment
Pelger et al., 1998[71]	Olpadronate 4 mg intravenously	28	Biochemical and clinical response	Decrease in urinary hydroxyproline excretion; decrease in bone pain in 75% patient	Trial not statistically powered to detect clinical benefit
Heidenreich et al., 2001[72]	Clodronate IV 300 mg for 8 days then 1600 mg daily	85	Pain and analgesia use, performance status and mobility	Decrease in pain and performance status	Nonrandomized study
Heidenreich et al., 2002[73]	Ibandronate 6 mg intravenously	25	Pain reduction using visual analog scale	Significant reduction in pain score in 92%, 39% pain free	Increase in Karnofsky index paralleled decrease pain
Saad et al., 2002[53]	Zoledronic acid 4mg vs. 8 mg vs. placebo	643	Skeletal related events (SRE), time to first SRE, pain	Reduction in proportion of patients with SRE's, non-significant reduction in time to SRE's, reduction in pain	Renal toxicity with zoledronic acid 8 mg, therefore, discontinued
Ernst et al., 2002[7]	Mitoxantrone+ prednisone+ clodronate vs. Mitoxantrone+ prednisone alone	208	Assessment of quality of life and health resource utilization	Non-significant decrease in pain	Multicentre trial

Trial	Compound/ Dose	Patients	Endpoints	Outcome	Comments
Smith et al., 2003[54]	Zoledronic acid 4 mg vs. placebo	106	Percent change (from baseline to 1 year) in bone mineral density of the lumbar spine	Mean bone density increased by 5.6% in treatment arm, decreased by 2.2% in controls	Trial in patients with nonmetastatic prostate cancer receiving androgen deprivation therapy

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