

Factors regulating the growth of metastatic cancer in bone

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Abstract

Metastatic tumor cells can interfere directly with the function of bone cells involved in normal bone remodeling or indirectly by influencing the behavior of hematopoietic, stromal and other cells in bone marrow that interact with bone cells. Recent studies of metastatic cancer have revealed that tumor cells interact closely with vascular endothelial cells, basement membrane and bone marrow stromal cells through cell surface proteins or by releasing factors which affect the function of these cells. Bidirectional interaction between marrow cells and tumor cells can give the latter a selective advantage for growth in bone which can lead to the destruction of or to increased production of bone matrix. Understanding of the mechanisms involved in tumor metastasis and growth in bone has increased in recent years, and in this review we shall describe current knowledge of these mechanisms and of the predilection of certain types of cancers to metastasize to bone, their growth in the bone microenvironment and interactions between them and bone cells. Because metastatic breast cancer has been studied more than any other, we shall focus on it as a representative example, although the general principles apply to other types of cancer and to myeloma.

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Bone modeling and remodeling

A firm grasp of the sequence of cellular events involved in the process of bone modeling and remodeling is essential for understanding the effects of tumor cells on the skeleton, but it is outside the scope of this review to describe these in detail here. However, because we shall be describing a number of models in which growing animals are used to examine the effects of tumors on the skeleton, bone modeling and aspects of bone remodeling relevant to the effects of malignancy will be covered briefly.

Long bones are sculpted from a cartilage analogue formed in limb buds during embryonic development and increase in length through endochondral ossification in which cartilage is calcified and subsequently replaced by bone matrix near the ends of the bones (reviewed by Panganiban *et al.* 1997, Shubin *et al.* 1997). They increase in width by periosteal matrix apposition by osteoblasts, and the marrow space is increased in diameter in proportion to the length of the growing bone by endosteal resorption of cortical bone by osteoclasts. New cancellous

bone is laid down rapidly following resorption of calcified cartilage at the growth plate, but most of it is quickly removed by osteoclastic resorption to maintain a medullary cavity as the bone grows. Mutations in genes regulating limb bud development lead to dwarfism or reduced numbers of skeletal elements, while defects in osteoclast generation or function result in build up of cancellous bone within the medullary cavity - the hallmark of osteopetrosis (reviewed by Mundlos & Olsen 1997, Boyce *et al.* 1999). Metastases to long bones of growing rodents used in experimental studies of the effects of tumors on the skeleton can interfere with normal bone modeling and endochondral ossification, and intervention therapy, such as bisphosphonates, given to inhibit bone resorption in such studies can result in a build up of unresorbed bone, similar to that seen in osteopetrosis (Sasaki *et al.* 1995).

Cancellous (also called trabecular) bone remains at the ends of long bones in humans after epiphyseal closure and, like cancellous bone in the axial skeleton, is remodeled throughout life. Bone remodeling begins on bone surfaces with a team of osteoclasts resorbing a trench of bone

Table 1 Agents released by tumor cells that stimulate bone resorption

Colony stimulating factors (CSFs)
Epidermal growth factor (EGF)
Interleukin-1 (IL-1)
Interleukin-6
Oxygen-derived free radicals
Platelet-derived growth factor (PDGF)
Prostaglandins
Parathyroid hormone (PTH)
Parathyroid hormone related protein (PTHrP)
Tumor necrosis factor α (TNF α)
Tumor necrosis factor β (TNF β ; lymphotoxin)
Transforming growth factors α and β (TGF α , TGF β)

approximately 60 μm deep. Osteoblasts then lay down new bone matrix in the trench in a process with many similarities to the repair of damaged sections of roadways. The two processes of resorption and formation are coupled temporally in this site-specific manner and it is estimated that there are about one million bone remodeling units in the normal adult skeleton at any time. The primary function of bone remodeling is thought to be the removal of worn-out sections of bone that have become weakened with age. The number of bone remodeling units is increased in conditions such as estrogen deficiency, hyperparathyroidism and Paget's disease, in which bone turnover is increased.

Osteoclasts are derived from precursors in the mononuclear-phagocyte lineage under the combined influence of macrophage colony-stimulating factor and the recently identified osteoclastogenic cytokine, known as RANK ligand, OPG ligand, TRANCE, and ODF (Anderson *et al.* 1997, Wong *et al.* 1997, Lacey *et al.* 1998, Yasuda *et al.* 1998). Osteoblasts are derived from the stromal cell lineage and in addition to laying down type I collagen, the major structural protein in bone, they secrete a number of growth factors into bone matrix (Hauschka *et al.* 1986). Hematopoietic tissue and stromal cell precursors persist at the ends of long bones and within the medullary cavities of vertebral bodies and co-operate with bone cells in regulating remodeling in these bones. As will be seen later, bone remodeling can significantly influence the function of metastatic tumor cells because the intense resorption of new bone matrix near growth plates and the resorption of remodeling bone elsewhere are associated with the release of growth factors from bone into the local microenvironment and these can promote tumor cell proliferation and osteolysis preferentially at these sites. In contrast, the diaphyseal cavity of long bones in humans is

normally devoid of trabecular bone and is filled with fatty marrow. Metastatic tumor deposits are found much less frequently at these sites than in the axial skeleton or the ends of long bones, presumably because there is less bone remodeling on the diaphyseal endosteal surfaces than at these other sites. Although these differences in bone volume and thus in bone remodeling rates between diaphyseal long bones and cancellous bone sites may account for the increased occurrence of metastases in the latter, they do not explain why metastases may occur in particular vertebrae, for example, while sparing others. This is likely to be the result of the interplay among a variety of factors, including blood flow rates, relative vascularity and bone remodeling rates at particular sites in the skeleton at the time tumor cells are circulating in the blood. However, we are unaware of any studies that have examined this aspect of bone metastasis specifically.

Local effects of tumor cells on bone

Some malignant tumours, such as breast, lung and prostate, have a predilection to spread to bone and typically cause osteolytic (breast and lung), osteoblastic (prostate) or mixed osteolytic and osteoblastic metastases (Mundy & Martin 1993). In addition to these solid tumors, myeloma typically causes extensive bone destruction and hypercalcemia (Mundy 1995). In all of these circumstances, the bone is resorbed by osteoclasts stimulated by tumor cell products, rather than by the tumor cells (Francini *et al.* 1993, Taube *et al.* 1994, Yoneda *et al.* 1994, Mundy & Yoneda 1995). Malignant tumors can also cause humorally mediated hypercalcemia by releasing factors, such as parathyroid hormone-related hormone (PTHrP), that act systemically to increase bone resorption and enhance renal tubular reabsorption of calcium. This subject has been reviewed elsewhere recently (Guise & Mundy 1998), and will not be covered here.

Bone metastases can cause bone pain, pathologic fractures, nerve compression syndromes (especially in myeloma) and hypercalcemia mainly as a result of the effects of growth factors, cytokines and hormones released into the bone marrow around them (Table 1). These act in a number of ways to increase osteoclast numbers and activity. For example, interleukin (IL)-6, which is released by myeloma cells, promotes the proliferation of early osteoclast precursors. However, it does not enhance fusion of the precursors to form osteoclasts and has only weak stimulatory effects on osteoclasts. In contrast, IL-1, which is produced by some solid tumors (Sato *et al.* 1988), stimulates osteoclast formation and activity (Uy *et al.* 1995), in part by prolonging their life spans (Jimi *et al.* 1998) through prevention of apoptosis (Hughes *et al.* 1994). PTHrP, which is released by many tumor cell types (Danks *et al.* 1989, Asa *et al.* 1990, Dunne *et al.* 1993), has

effects on osteoclasts similar to those of IL-1 although, unlike IL-1, it does not stimulate the production of granulocyte-macrophage colony-forming units from which osteoclast precursors arise (De La Mata *et al.* 1995). Release of PTHrP is often associated with hypercalcemia because it also enhances renal tubular reabsorption of calcium.

Predilection for breast cancer to metastasize to bone

The predilection for breast cancer to metastasize to bone was described more than 50 years ago by Walther (1948). He found in an autopsy study (in which the confounding influence of chemotherapy was not a factor) that 64% of 186 patients who died of breast cancer had metastases to bone. Two more recent studies reported that 71% of 707 (Cifuentes & Pickren 1979) and 62% of 1060 (Weiss 1992) breast cancer patients had bone metastases at autopsy, suggesting that chemotherapy has not affected the predilection for or frequency of metastasis to bone. Furthermore, in another study in which 69% of 587 patients dying of breast cancer had bone metastases (Coleman & Rubens 1987), bone was the most common site of first distant relapse. Thus, bone metastasis is a common complication of breast cancer and one for which only palliative therapy is presently available.

Two major factors determine the dissemination of cancers to distant organs: the biological properties of the cancer cells and the environment at the metastatic site. Metastasis to and growth of tumor cells in distant organs involve multiple and complex steps (Fidler 1990, Liotta 1992). Cancer cells with a high predilection for metastasis to bone must have properties not present in tumors that rarely spread to bone. These could include production of proteolytic enzymes, angiogenic factors, autocrine growth-stimulating factors, increased expression of growth factor receptors, temporal and spatial expression of cell adhesion molecules (CAMs) and resistance to host immune surveillance. However, most metastatic cancers are likely to possess many of these properties, and thus other characteristics must account for the preferential colonization of bone by breast cancer.

Metastatic cancer cells enter bone mainly through nutrient arteries and these communicate with a sinusoidal network in the bone marrow (DeBruyn 1981, Yoneda 1997) rather than with a capillary system found in most solid organs. They express CAMs to establish contact with sinus endothelial cells and secrete proteolytic enzymes to degrade the endothelial wall and pass into the bone marrow compartment. Certain cancer cells attach preferentially to endothelial cells in particular organs (Auerbach *et al.* 1987), suggesting a contributory role of endothelial cells in organ preference. Furthermore, protease secretion

by cancer cells is also influenced by target organs (Nakajima *et al.* 1990). The particular cell-cell adhesion molecules and proteases involved in breast cancer cell attachment and invasion in bone are as yet unidentified.

There are numerous types of cells in bone marrow involved in the maintenance of immune and inflammatory responses and in blood cell homeostasis. The activities of these cells, which include lymphocytes, plasma cells, macrophages and erythroid and myeloid precursors, are regulated by a variety of cytokines and growth factors and complex cytokine growth factor networks. Breast cancer cells migrating into the bone marrow are exposed to cells producing these cytokines and growth factors, and it is likely that they interact with one another to enhance tumor cell growth and activity. The progression of osteolytic metastases requires the establishment of functional interactions between metastatic breast cancer cells and osteoclasts. These interactions could be mediated by direct cell-cell contact and/or production of soluble stimulators of osteoclast activity (Yoneda *et al.* 1994, Mundy & Yoneda 1995, Yoneda 1998).

Several genes have been identified recently that may contribute to the metastatic potential of breast cancer. An anti-metastatic gene (NM23) was originally cloned from low metastatic murine melanoma cells using subtractive hybridization techniques (Steege *et al.* 1988) and high expression of it is associated with a good prognosis in breast cancer patients (Hennessy *et al.* 1991, Hirayama *et al.* 1991). Decreased expression of a metastasis suppressor gene called KAI1 has been observed in metastatic human prostate cancer cells (Dong *et al.* 1995), while a metastasis-promoting gene named MTA1 has been cloned from rat mammary adenocarcinoma cells using differential hybridization (Toh *et al.* 1994). Since the protein products of these genes have not yet been characterized, it is not known if they play a role in breast cancer metastasis to and organ selectivity for bone.

The bone microenvironment

The observation that most cancers exhibit target organ preference when they disseminate was first reported by Paget (1889) in a study of autopsy records of 735 women who died of breast cancer. He found that the highest numbers of metastases were in the ovaries, followed by the skeleton. On the basis of these observations, he proposed the 'Seed and Soil' theory that the microenvironment of the organs to which cancer cells spread serves as a fertile soil for their growth. This hypothesis has been widely accepted as a basic principle in the field of cancer metastasis (for review see Rusciano & Burger 1992), and we believe it is particularly relevant to bone.

During bone formation, osteoblasts lay down a variety of growth factors which become incorporated into bone

matrix along with type I collagen (Hauschka *et al.* 1986). These are released in active form into the marrow when bone matrix is degraded during osteoclastic resorption (Pfeilschifter & Mundy 1987). Many of these growth factors, which include transforming-growth factor (TGF) β , fibroblast growth factors (FGF), insulin-like growth factors (IGF) and bone morphogenetic proteins (BMP), could stimulate the growth of metastatic cancer cells in the marrow. Among these, IGFs, whose concentrations in bone are higher than those of other growth factors (Hauschka *et al.* 1986), promote the growth of human breast cancer MDA-MB-231 cells (Yoneda *et al.* 1995). Furthermore, the mitogenic activity of culture supernatants from resorbing bone on MDA-MB-231 cells was decreased by the addition of neutralizing antibodies to the IGF-I receptor (Yoneda *et al.* 1995). TGF β has been shown to stimulate the proliferation of Walker 256 carcinosarcoma cells that metastasize to bone (Orr *et al.* 1995).

Further evidence that the target organ host environment influences the metastatic potential of cancers is the observation that some cancers increase their meta-static and organ-preferential properties by successive *in vivo* passages through target organs. For example, murine melanoma B16 cells with low metastatic potential become highly metastatic to lung (B16F10) and liver (B16L8) after repeated selection and culture from pulmonary (Hart & Fidler 1981) or hepatic (Tao *et al.* 1979) metastatic foci respectively. B16L8 cells respond specifically to growth factors from hepatocytes, whereas B16F10 do not (Sargent *et al.* 1988). Other examples include human colon cancer cells which developed high metastatic ability and organ selectivity for the liver (Morikawa *et al.* 1988) following repeated passage, and this was associated with expression of greater numbers of functional receptors for TGF α and hepatocyte growth factor than colon cancer cells with low metastatic potential (Fidler 1995). Although it is unclear from these studies whether the multiple *in vivo* passages resulted in enrichment of highly metastatic and organ-preferential subpopulations of cancer cells or in acquisition of metastatic ability and organ preference, they demonstrate that the metastatic potential and behavior of cancer cells can be altered by specific organ environments.

Growth factors present in bone could also contribute to the bone preference of breast cancer by being chemotactic for tumor cells. For example, culture supernatants of resorbing bone (Orr *et al.* 1979) and type I collagen and its fragments (Mundy *et al.* 1981) released during bone resorption stimulate chemotaxis of breast cancer cells in a Boyden chamber assay. Thus, following entry to the bone marrow space, breast cancer cells might migrate preferentially to resorbing bone surfaces in response to an increasing local concentration gradient of bone products, such as type I collagen, and then be exposed to high local

levels of bone-derived growth factors that could promote their proliferation.

Although bone provides a favorable environment for proliferation of metastatic breast and other cancer cells, this alone cannot account for the special propensity of these cells to thrive in it, since other metastatic cancer cells passing through bone are likely to be exposed to the same influences. For example, as outlined in detail later, the release of factors such as PTHrP by breast cancer cells could stimulate osteoclastic resorption around them, resulting in the release of more growth factors from bone, thus establishing an up-regulatory cycle between these cell types.

Experimental approaches to the study of breast cancer metastasis to bone

Detailed study of the metastatic behavior of cancers and of the complex interactions between cancer cells and host cells requires the development of good animal models. The ideal model would be one in which cancer develops spontaneously in the organ of interest and metastasizes to bone. However, these models are extremely difficult to establish. Thus, few are available (Orr *et al.* 1995) and there are no spontaneous models of breast cancer metastatic to bone.

This problem can be partly circumvented in models in which cancer cells are injected directly into the blood stream. The cellular and molecular mechanisms of organ preference of metastatic tumors can be studied in such an experimental model because the steps involved before cancer cells reach their preferential target organs are likely to be non-specific and independent of organ selectivity. To this end, we modified the model originally described by Arguello *et al.* (1988) to develop an animal model of bone metastasis of human breast cancer in nude mice (Yoneda *et al.* 1994). We injected the human breast cancer cell line, MDA-MB-231, into the left cardiac ventricle of female nude mice and found that these cells preferentially cause osteolytic bone metastases and rarely form metastases in other organs. More recently, we observed that these animals exhibit osteolytic metastases to the jaw, including mandible, maxilla, and zygomatic arch (Sasaki *et al.* 1998).

We monitored the development and growth of osteolytic lesions by serial radiography and quantitated the size of the lesions by computer-assisted image analysis. Animals rarely became hypercalcemic, but frequently developed cachexia and occasionally paraplegia, due to vertebral metastases. Replacement of the bone and marrow at the ends of long bones with metastatic breast cancer cells was confirmed histologically and numerous multinucleated osteoclasts were seen adjacent to cancer cells on the endosteal bone surface (Sasaki *et al.* 1995). Similar findings have been reported using the human

melanoma cell line, A375 (Nakai *et al.* 1992, Hiraga *et al.* 1995).

There are several other animal models of bone metastasis (Orr *et al.* 1995). These include the Walker 256 carcinosarcoma, which metastasizes spontaneously to bone after intramuscular inoculation (Kostenuik *et al.* 1992), and the human PC3 prostate cancer, which metastasizes to vertebrae in nude mice following tail vein injection if the vena cava is compressed beforehand to force the flow of blood and the injected tumor cells into the vertebrae via Barton's vertebral venous complex (Shevrin *et al.* 1988).

Because metastasis is a complex multistep process, certain aspects of it cannot be studied in detail using *in vivo* approaches and reproducible, quantitative and convenient *in vitro* assays are required. *In vitro* models for attachment to extracellular matrix, invasion, chemotactic migration and matrix metalloproteinase (MMP) production have been developed (Mareel *et al.* 1991a) for study of the general steps of cancer metastasis, but *in vitro* models for the study of metastasis to bone specifically are difficult. Currently, the effects of cancer products on bone resorption or osteoclast formation are generally studied using organ cultures of radiolabeled fetal rat long bones or mouse calvariae or bone marrow cells cultured in the presence of the culture supernatants of cancer cells. Culture supernatants of bone or osteoblasts can be assessed for their effects on cancer cell proliferation and production of proteases, cytokines and growth factors which promote osteoclastic bone resorption. However, additional *in vitro* assays which can examine other specific components of the *in vivo* processes of bone metastasis are needed to further advance the study of cancer metastasis to bone.

Growth of metastatic cancer and the bone microenvironment

One way to determine whether factors released during bone resorption promote the growth of metastatic cancer cells in bone is to inhibit bone resorption before or after tumor cells metastasize to bone. To test this hypothesis, we inoculated breast cancer cells into the left cardiac ventricle of nude mice and treated the mice with the bone resorption-inhibiting bisphosphonate, risedronate. We used three different protocols, in which risedronate was given before, simultaneously with or after breast cancer cell inoculation. In these experiments, risedronate either prevented the development of new osteolytic bone metastases or decreased the progression of those already established. Importantly, histomorphometric analysis revealed that, in risedronate-treated mice, the tumor burden in bone was markedly decreased compared with that in untreated mice (Sasaki *et al.* 1995). These findings and a similar study in rats (Hall & Stoica 1994) suggest that

reduced growth factor release from bone due to bisphosphonate-induced inhibition of osteoclastic bone resorption may impair metastatic breast cancer growth in bone. Of note, in some experiments, we observed that metastases of MDA-231 cells to liver and adrenal gland were increased in bisphosphonate-treated mice. Similar experimental findings have been reported by Kostenuik *et al.* (1993) and Stearns & Wang (1996). However, the clinical significance of these observations for humans with metastatic cancer has yet to be elucidated.

To further test our hypothesis that tumor cell metastasis and proliferation are enhanced by the products of bone resorption, we increased bone resorption by injecting IL-1 locally over the calvaria of nude mice for 3 days according to the methods described by Boyce *et al.* (1992). Breast cancer cells were inoculated into the left cardiac ventricle the following day, and the mice were examined for the development of cancer metastases in the calvariae at 4 weeks after cell inoculation. Cancer cells rarely metastasize spontaneously to calvariae in this model and we speculated that this was due to a low basal level of bone turnover in calvariae compared with other bones. Metastatic tumor deposits were clearly visible in the calvariae of IL-1-treated mice (Sasaki *et al.* 1994), and radiologic and histologic examination revealed that these were osteolytic. In contrast, no metastatic tumor deposits were detected in calvariae of phosphate-buffered saline (PBS)-treated mice. Furthermore, treatment of mice with risedronate prior to IL-1 injections, to suppress the IL-1-induced increase in bone turnover, significantly reduced metastasis formation (Sasaki *et al.* 1994).

To further study the effects of bone-derived factors on tumor cell growth, we added the supernatants from resorbing neonatal mouse calvariae to cultures of breast cancer cells and found that they strongly increased breast cancer cell proliferation (Yoneda *et al.* 1995). However, no stimulation of breast cancer cell proliferation was observed when bone resorption was inhibited *in vitro* by risedronate. Furthermore, the growth-stimulating effects of the resorbing bone culture supernatants on the tumor cells was markedly impaired by neutralizing antibodies to IGF-I receptors (Yoneda *et al.* 1995).

Metastatic cancer cell growth might also be affected by osteoblasts in bone. For example, the culture supernatants of osteoblasts increase both chemotactic migration and matrix metalloproteinase production by breast cancer cells *in vitro* (Giunciuglio *et al.* 1995). However, further studies are needed to fully examine the role of osteoblasts in cancer colonization in bone.

General cancer cell properties involved in bone metastasis

Cell adhesion molecules have been shown to have key roles in several critical steps involved in cancer cell invasion and metastasis. In normal cells they mediate cell-

to-cell and cell-to-substratum communications, and cancer cells may decrease or increase their expression of CAMs to assist them to leave the primary site and to attach and proliferate at a fertile metastatic site (Albelda & Buck 1990).

Integrins are the most abundantly expressed CAMs (Haynes 1992). They have been implicated in cancer dissemination (Juliano & Varner 1993) by mediating cancer cell attachment to vascular endothelial cells and to underlying matrix proteins, such as laminin and fibronectin (Albelda & Buck 1990). Expression of the $\alpha_v\beta_3$ integrin (vitronectin receptor) on the surface of human melanoma cells increases when they bind to and invade the basement membrane matrix, matrigel (Seftor *et al.* 1992), and neutralizing antibodies to $\alpha_v\beta_3$ integrins inhibit tumor growth and invasion *in vivo* (Brooks *et al.* 1994). However, their role in bone metastasis has not been studied, as yet.

Laminin is a major component of basement membrane that has been implicated in malignancy (Menard *et al.* 1997). We have carried out two studies that suggest a role for laminin in bone metastasis. A multimeric anti-laminin peptide, YIGSR, reduced the growth of osteolytic metastases of a human melanoma cell line, A375 (Nakai *et al.* 1992), and of a B-cell lymphoma cell line, MH-95 (Michigami *et al.* 1998) in nude mice inoculated with these tumors cells. This peptide has been reported to inhibit angiogenesis (Iwamoto *et al.* 1996). Thus, these studies implicate angiogenesis in the development of bone metastases.

E-cadherin (Uvomorulin) is a 120 kDa cell surface glycoprotein involved in calcium-dependent epithelial cell-cell adhesion which appears to play a suppressive role in cancer invasion and metastasis (Takeichi 1993). E-cadherin expression in cancer cells is reversibly modulated according to culture conditions *in vitro* and environmental factors *in vivo* (Mareel *et al.* 1991b). Its expression in human tumors correlates inversely with breast cancer metastasis (Oka *et al.* 1993) and is increased in subpopulations of MCF-7 breast cancer cells with reduced invasiveness, but is undetectable in the highly invasive MDA-MB-231 breast cancer cells (Sommers *et al.* 1991). Most studies to date have examined only the inhibitory effects of E-cadherin on invasiveness of tumor cells at the primary site and few have examined the possible role of E-cadherin in cancer metastasis to distant organs, including bones. To examine this question, we stably transfected MDA-MB-231 cells with E-cadherin and found markedly fewer osteolytic metastases in nude mice inoculated with the transfected cells compared with controls (Mbalaviele *et al.* 1996), suggesting that increased cell-cell adhesiveness may reduce their capacity to grow in bone.

In a murine myeloma model associated with massive osteoclastic bone destruction, we have found that the cell-cell interactions between marrow stromal cells and myeloma cells mediated via VCAM-1 and $\alpha_4\beta_1$ integrin VCAM, respectively, are critical for myeloma cell production of osteoclast-activating factor(s) (Michigami *et al.* 1997). Disruption of these interactions could be an effective therapeutic intervention for the treatment of the devastating bone destruction seen typically in myeloma.

Organ-selective adherence of cancer cells

A number of investigators have attempted to examine organ selectivity of malignant cells. For example, Kieran & Longnecker (1983) and Netland & Zetter (1984) showed that ^{57}Cr -labeled cancer cells bound selectively to fresh cryostat sections of particular host organs. In more convincing studies, Nicholson (1988) showed that cells can exhibit preference for specific sites. Following injection of tumor cells into the tail vein of mice, he removed cells that formed brain or lung metastases from these sites and repeatedly reinjected them into other mice. He thus established two sub-clonal cell lines of the B16 murine melanoma. One spread preferentially to brain and adhered selectively to brain-derived endothelial cells, while another sub-clone metastasized preferentially to the lungs. Furthermore, Haq *et al.* (1992) found that the rat Dunning prostate carcinoma cell line that disseminates to bone adheres preferentially to cultures of bone marrow stromal cells enriched for endothelial cells.

Highly invasive cancer cells produce large amounts of MMPs that comprise a family of at least eight zinc-dependent endopeptidases with related structures, but different substrate specificities. Increased expression of MMPs correlates with the development of invasion and metastasis in human breast, colon, stomach, thyroid, lung and liver cancers (Seftor *et al.* 1992, Zucker *et al.* 1993). It is likely that individual cancer cells utilize several MMPs, as well as other classes of destructive enzymes, to cross the various tissue boundaries they encounter as they invade and metastasize.

We have examined MMP expression by MDA-MB-231 and found that when the cells were cultured on plastic they produced 92 kDa (MMP-9) and 72 kDa (MMP-2) MMPs in latent forms. However, when they were cultured on bone extracellular matrix laid down by osteoblasts the cells released active forms of both these MMPs, while others cultured on laminin, fibronectin, type I collagen, matrigel and poly-L-lysine released latent forms of MMPs (Yoneda *et al.* 1997).

Cancer invasiveness and metastatic capacity is determined not only by expression levels of MMPs, but also by those of the corresponding tissue inhibitors of

matrix metalloproteinases (TIMPs), at least two of which are distributed ubiquitously (Liotta 1992). TIMPs function as metastasis suppressors, and the invasive capacity of cancer cells is likely to depend on the balance between MMP and TIMP production. Transfection of tumor cells with the TIMP-2 gene partially suppressed their invasion and metastasis in animals, and injections of recombinant TIMP-2 blocked metastasis of tumor cells (DeClerck *et al.* 1992). We transfected MDA-MB-231 cells with TIMP-2 cDNA and, in a preliminary experiment, observed that mice inoculated with these cells had fewer osteolytic metastases than mice inoculated with MDA-MB-231 cells transfected with the empty vector (Yoneda *et al.* 1997).

PTHrP and bone metastasis

PTHrP is produced by a variety of normal tissues, including breast, skin, placenta, and uterine and vascular smooth muscle (Philbrick *et al.* 1996), and is also produced by many types of malignant tumors, both at the primary site and at sites of metastasis (Danks *et al.* 1989, Asa *et al.* 1990, Dunne *et al.* 1993). In a study examining its expression by breast cancer cells, PTHrP was detected immunohistochemically in 56% of 155 primary breast tumors from normocalcemic women and its expression correlated with the development of bone metastases (Bundred *et al.* 1992). However, PTHrP expression in primary breast tumors does not appear to correlate with standard prognostic factors, recurrence or survival (Southby *et al.* 1990, Bundred *et al.* 1992, Liapis *et al.* 1993). Its expression by breast cancer cells appears to increase when the tumor cells metastasize to bone. For example, it has been detected by immunohistochemistry (Powell *et al.* 1991, Kohno *et al.* 1994) and *in situ* hybridization (Vargas *et al.* 1992) in 80-90% of breast cancer metastases in bone, compared with only 38% in lung (Kohno *et al.* 1994) and 17% (Powell *et al.* 1991) in other non-bone sites. These findings suggest that PTHrP expression by breast cancer cells increases when they metastasize to bone and support a major potential role for tumor-produced PTHrP to mediate the increased bone resorption around osteolytic breast metastases.

In view of these clinical observations, we have used the nude mouse model of bone metastasis and MDA-MB-231 cells which produce low amounts of PTHrP constitutively *in vitro* to further examine the role of PTHrP in the development of bone metastases. We increased PTHrP production by these cells by transfecting them with the cDNA for human preproPTHrP and inoculated them into nude mice. This resulted in an increase in the number of osteolytic metastases *in vivo* in the mice bearing the transfected cells compared with controls (Guise *et al.* 1994). Furthermore, treatment of mice with monoclonal antibodies directed against the 1-34 region of PTHrP prior

to inoculation with parental MDA-MB-231 cells dramatically reduced the number and size of osteolytic lesions compared with controls (Guise *et al.* 1996). Treatment with the PTHrP antibody also decreased the rate of progression of established osteolytic metastases compared with controls (Yin *et al.* 1995).

These data strongly suggest that PTHrP expression by breast cancer cells promotes the development and progression of metastases in bone, but they do not provide an explanation for the clinical observation that PTHrP expression by breast cancer cells increases when the cells metastasize to bone. Several candidate factors have been identified that could promote increased release of PTHrP from breast cancer cells in bone. For example, epidermal growth factor (EGF) stimulates PTHrP expression by a keratinocyte (Allinson & Drucker 1992) and a mammary epithelial cell line (Sebag *et al.* 1994), while TGF α enhances PTHrP expression in a human squamous cell carcinoma of the lung (Burton *et al.* 1990). IL-6, tumor necrosis factor (TNF), IGF-I and IGF-II increased the production of PTHrP *in vitro* by a human squamous cell carcinoma (Rizzoli *et al.* 1994). TGF β , which is present in high concentrations in bone matrix (Hauschka *et al.* 1986) and is expressed by some breast cancers (Dublin *et al.* 1993) and cancer-associated stromal cells (van Roozendaal *et al.* 1995), has been shown to enhance secretion of PTHrP in a renal and squamous cell carcinoma (Kiryama *et al.* 1992, Merryman *et al.* 1994). TGF β is stored in bone and is released and activated during osteoclastic bone resorption (Pfeilschifter & Mundy 1987). It increases PTHrP expression by MDA-MB-231 cells *in vitro* and thus could potentiate the development of bone metastases by increasing PTHrP production by these cells in bone metastases.

To examine this possible role for TGF β , we transfected MDA-MB-231 cells with a cDNA encoding a TGF β type II receptor lacking a cytoplasmic domain (T β RII Δ cyt) and inoculated the cells into the left ventricle of nude mice. TGF β binds to this receptor, but signal transduction is not initiated and, thus, the receptor acts in a dominant-negative fashion to block the biologic effects of TGF β . PTHrP secretion did not increase in response to TGF β in stable clones expressing T β RII Δ cyt compared with controls of untransfected MDA-MB-231 cells or those transfected with the empty vector. Mice inoculated with MDA-MB-231 cells expressing T β RII Δ cyt had fewer and smaller osteolytic lesions than control mice given parental or empty vector-transfected cells (Yin *et al.* 1999). Reversal of the dominant-negative signaling blockade by expression of a constitutively active TGF β type I receptor in the breast cancer cells increased tumor production of PTHrP, caused marked enhancement of osteolytic bone metastasis (Figs 1 and 2) and decreased survival. To determine if the effects of TGF β to increase

Table 2 Factors produced by tumor cells that stimulate bone formation

Bone-derived growth factor (BDGF, β 2 microglobulin)
Bone morphogenetic proteins (BMPs)
Endothelin 1
Fibroblast growth factors (FGF acidic and basic)
Insulin-like growth factors (IGF-I and II)
Interleukin-1 (IL-1)
Macrophage-derived growth factor (MDGF)
Platelet-derived growth factor (PDGF)
Prostaglandins
Transforming growth factor β (TGF β)
Tumor necrosis factor α (TNF α)
Tumor necrosis factor β (Lymphotoxin)
Urokinase (Urinary Plasminogen Activator, uPA)

bone metastases were mediated by PTHrP, the MDA-MB-231 cells which expressed the dominant-negative TGF β

type II receptor were transfected with the cDNA for PTHrP. This resulted in a marked increase in tumor PTHrP production and accelerated bone metastases. To determine if the effects of TGF β to promote bone metastases were mediated by PTHrP, the MDA-MB-231 cells which expressed the dominant-negative TGF β type II receptor were transfected with the cDNA for PTHrP (Yin *et al.* 1999). This resulted in constitutive tumor PTHrP production and accelerated bone metastasis. These data demonstrate an important role for TGF β in the development of breast cancer metastasis to bone, via the TGF β receptor-mediated signaling pathway in tumor cells, and suggest that the bone destruction is mediated by PTHrP. Thus, these findings support the hypothesis that TGF β released from bone during metastasis-stimulated resorption maintains an up-regulatory loop in which it promotes PTHrP secretion by breast cancer cells. This in turn promotes more osteoclastic bone resorption and more release of TGF β from bone, thus inducing further tumor growth and bone destruction.

Although loss of TGF β receptor function (Markowitz *et al.* 1995) or its signaling molecules (Eppert *et al.* 1996, Hahn *et al.* 1996, Takaku *et al.* 1998, Zhu *et al.* 1998) has

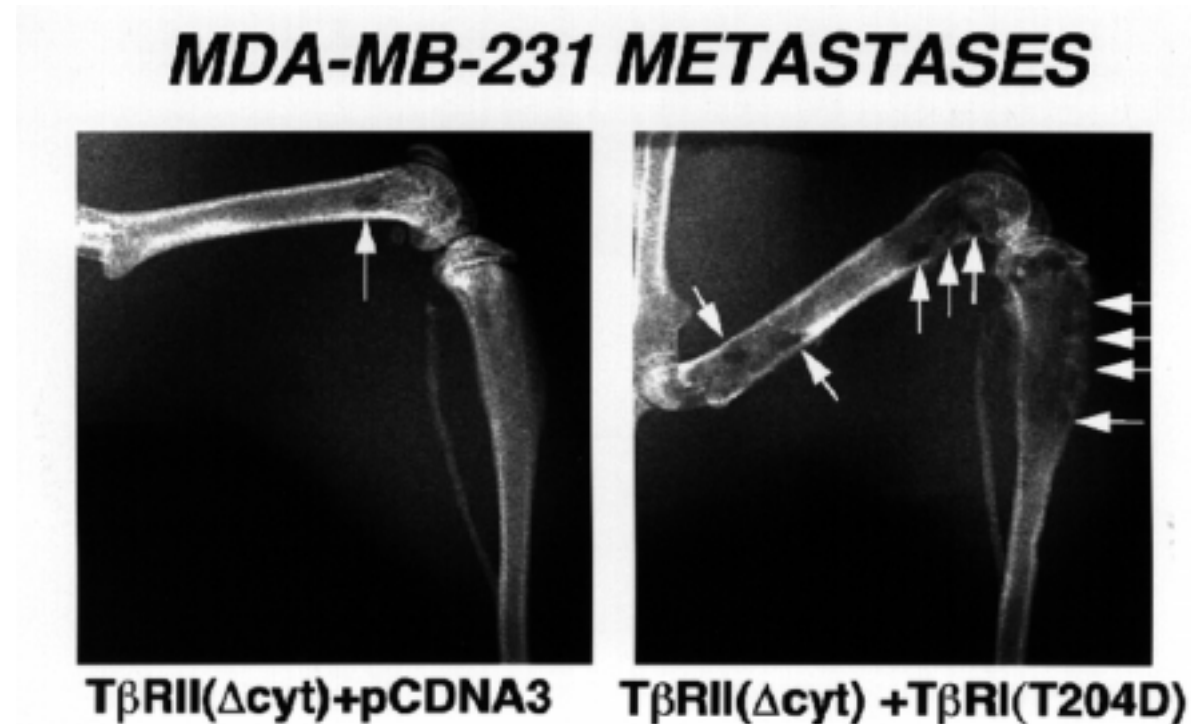


Figure 1 Representative radiographs of hindlimbs from nude mice bearing MDA-231 breast cancer cells transfected with a dominant-negative TGF β receptor (T β RII Δ cyt) + an empty vector (pcDNA3.1zeo; pcDNA3) or with a T β RII Δ cyt + a constitutively active TGF β receptor (T β RI(T204D)) 28 days after tumor inoculation. Increased numbers of osteolytic lesions (indicated by the arrows) were seen in the mice inoculated with tumor cells transfected with the T β RII Δ cyt + T β RI(T204D) transfected cells. Reproduced with permission from Yin *et al.* 1999 *Journal of Clinical Investigation* **103** 197-206.

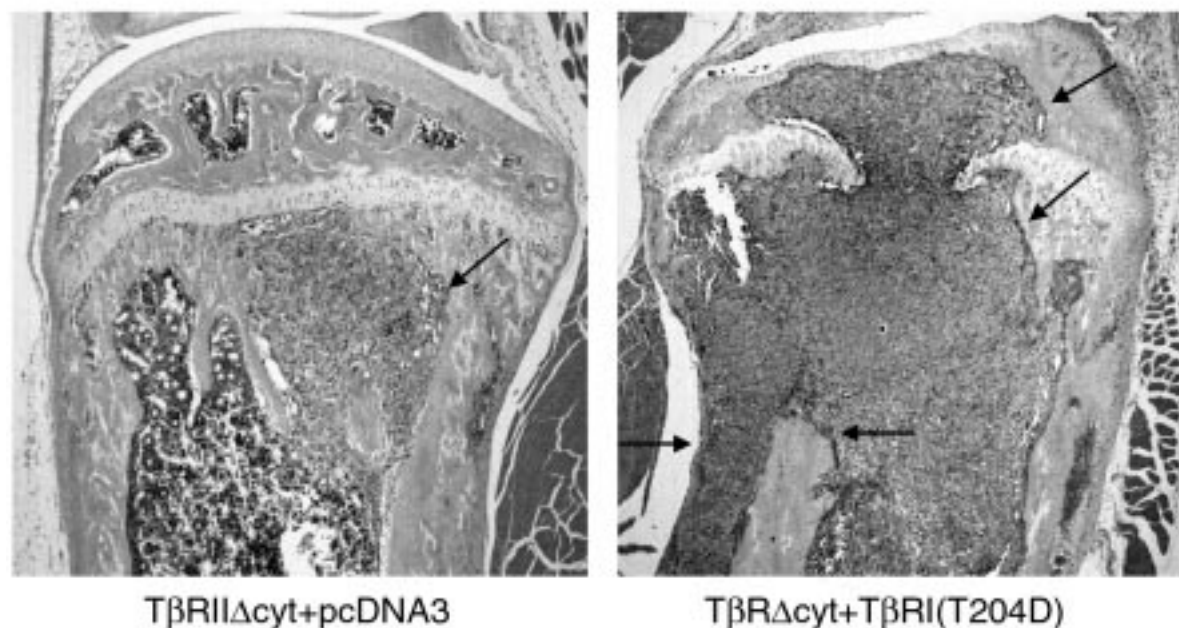


Figure 2 Bone histology from the proximal tibiae of representative mice bearing either MDA-231 cells transfected with either $T\beta RI\Delta cyt + T\beta RI(T204D)$ or $T\beta RI\Delta cyt + pcDNA3.1zeo (pcDNA3)$. Tumor (arrows) filled much of the marrow cavity and replaced normal cellular elements in mice bearing $T\beta RI\Delta cyt + T\beta RI(T204D)$ tumors (right). There was significant loss of both cortical and trabecular bone in this group, and tumor has eroded through the growth plate of the tibia of this mouse. In contrast, sections from mice bearing control $T\beta RI\Delta cyt + pcDNA3.1zeo$ tumors (left) had small foci of tumor in the marrow cavity (arrow) with little bone destruction, as evidenced by intact trabecular and cortical bone. Reproduced with permission from Yin *et al.* 1999 *Journal of Clinical Investigation* **103** 197-206.

been associated with malignant progression (Massagué 1998), there is growing evidence that $TGF\beta$ may enhance tumor growth and invasion. For example, $TGF\beta$ induces an epithelial-mesenchymal transdifferentiation and an invasive phenotype (Miettinen *et al.* 1994, Caulin *et al.* 1995). Oft *et al.* (1998), using the same dominant-negative $TGF\beta$ type II receptor approach, demonstrated that $TGF\beta$ blockade decreased invasion and metastases in a mouse colon carcinoma and that several human carcinoma cell lines lost *in vitro* invasiveness when treated with neutralizing $TGF\beta$ antibodies. Thus, it appears that $TGF\beta$ signaling is required for both induction and maintenance of invasiveness *in vitro* and metastasis during late-stage tumorigenesis.

Metastatic prostate cancer

Prostatic cancer, like breast cancer, has a distinct predilection for metastasis to and growth within bone. Up to 70% of patients with advanced prostatic cancer have bone metastases, and most of these are osteoblastic. Metastases are found most frequently in lumbar vertebrae and pelvic bones following retrograde spread via Barton's vertebral venous plexus, and bone is the second most common

metastatic site after regional lymph nodes (Galasko 1981). However, diffuse skeletal involvement is relatively common, as are mixed osteoblastic and osteolytic lesions. The cancer cells appear to stimulate new bone formation by causing osteoblasts on fully calcified, 'quiescent' bone surfaces to lay down new matrix without preceding resorption and also by stimulating osteoblast precursors in the bone marrow to proliferate and lay down new bone matrix between pre-existing bone trabeculae (Valentin *et al.* 1980). Osteoblastic metastases are typically 'hot' on bone scan and, although osteosclerotic, may result in vertebral collapse and paraplegia because much of the new bone is woven with intrinsically low strength and because there may be concomitant osteolysis.

Normal and malignant prostatic cells express a host of growth factors (see Table 2) and some of their receptors (reviewed by Koutsilieris 1995), some of which, such as $TGF\beta$, BMPs, IGFs and FGFs, can stimulate osteoblast proliferation, while others, such as PTHrP, platelet-derived growth factor (PDGF) and $TGF\beta$, stimulate bone resorption (Iwamura *et al.* 1993). Furthermore, bone marrow stromal cells (Chackal-Roy *et al.* 1989) and, in particular, cells in the osteoblast lineage (Gleave *et al.* 1992), produce factors which are mitogenic for prostatic

cancer cells, indicating that there may be bi-directional interactions favoring the growth of tumor cells and osteoblasts in close proximity to one another.

In addition to these growth factors, prostatic cancer cells produce urokinase-type plasminogen activator (uPA) and endothelin-1 (Nelson *et al.* 1995) which may enhance the growth of osteoblasts at the metastatic site. Indeed, plasma levels of endothelin-1 are elevated in patients with metastatic prostatic cancer (Nelson *et al.* 1995). uPA appears to stimulate osteoblast proliferation by hydrolysing IGF-binding proteins and thus activating the growth factors (Koutsilieris *et al.* 1993). Furthermore, transfection of rat prostate cancer cells with full-length uPA cDNA promotes the growth of the tumor cells themselves and causes earlier development of metastases after intracardiac injection of the transfected cells compared with controls (Koutsilieris *et al.* 1993).

Although osteoblastic bone metastases are common in advanced prostate and breast cancer, the pathogenesis of the increased bone formation remains poorly understood, in part because there are few models of metastasizing osteosclerotic tumors. During study of the effects of a human breast cancer cell line, ZR-75-1, in our bone metastasis model, we discovered that these tumor cells cause osteoblastic metastases (Yin *et al.* 1998). We compared the effects on bone of these tumor cells with those of the human breast cancer line, MDA-MB-231 (MDA-231), which causes osteolytic metastases. ZR-75-1, MDA-231 or vehicle control (PBS) were inoculated into the left cardiac ventricle of nude mice. Histo-morphometric analysis of long bones from these animals demonstrated significantly greater bone volume in ZR-75-1-bearing mice compared with those bearing MDA-231 or treated with PBS. There was no significant difference in tumor volume between ZR-75-1- and MDA-231-treated animals. Osteoclast number per long bone section was greatest in MDA-231-bearing mice, but there was no significant difference between ZR-75-1 and PBS (Guise & Mundy 1998). To determine the mechanism responsible for the increased new bone formation in the ZR-75-1-bearing mice, ZR-75-1 cells were screened for factors known to stimulate new bone formation and compared with MDA-231. Of these factors (TGF β -1 and -2, BMP-2, -3, -4, -6, IGF-I and -II, prostate-specific antigen (PSA), uPA, and endothelin-1 (ET-1)), ET-1 was the only potential osteoblast-stimulating factor produced in greater amounts *in vitro* by ZR-75-1 cells compared with MDA-231 cells (Yin *et al.* 1998). PTHrP was produced by MDA-231 and not ZR-75-1 cells. To investigate whether ET-1 plays a role in the new bone formation stimulated by ZR-75-1 cells, the effect of ET-1 and ZR-75-1 conditioned medium was tested on new bone formation in neonatal mouse calvarial organ cultures. ZR-75-1 conditioned medium and synthetic ET-1 each stimulated osteoblast

proliferation and new bone formation *in vitro* in a dose-dependent manner, comparable to that stimulated by BMP-2 or FGF-2. In contrast, conditioned media from MDA-231 cells had no effect on new bone formation. New bone formation caused by both ZR-75-1 conditioned medium and ET-1 was inhibited by BQ-123, an endothelin A receptor antagonist. Finally, we have identified two other human breast cancer cell lines which secrete ET-1 and cause osteoblastic metastases *in vivo*. The preliminary data suggest that the effects of ET-1 on new bone formation are mediated via the endothelin A receptor.

The production of osteoclast-stimulating factors by prostate cancer cells could account for the osteolysis seen in some metastases. The expression level of these by tumor cells relative to that of osteoblast-stimulating factors is likely to determine whether individual metastases are osteolytic, osteoblastic or mixed. Recent studies have shown that PSA, a serine protease, homologous to the kallikrein family of proteases (Riegman *et al.* 1989) can cleave PTHrP and completely abolish its ability to stimulate cAMP production (Cramer *et al.* 1996). Thus, although PTHrP is produced by many prostate cancers, it could be inactivated in bone by PSA, thus allowing an osteoblast response to predominate in most metastases.

Summary

Clinically overt bone metastases are common in patients with advanced cancer and their presence is typically accompanied by a grave prognosis. Recent studies have identified a number of factors that regulate the proliferation and activity of metastatic cells and their interaction with bone cells after they have seeded in bone. However, despite advances in our understanding of the close interaction that takes place between cancer cells and bone cells, the specific molecular mechanisms by which cancer cells spread to and destroy bone or stimulate new bone formation remain poorly understood. Furthermore, therapeutic agents with proven efficacy to prevent or reverse metastatic bone disease have yet to be developed. Recent studies indicating that bisphosphonate therapy may have beneficial direct and indirect effects on tumor cell growth in bone suggest that the poor prognosis associated with metastatic bone disease could be ameliorated with intervention therapy, particularly if it can be demonstrated that therapy given early in the course of the disease prevents the development of metastases. Many of the factors produced in excess by tumor cells in bone are likely to be released locally in much lower concentrations by normal cells within bone marrow and to be involved in the regulation of bone remodeling. Thus, study of the effects of cancer cells on bone should not only benefit patients with cancer, but also improve our understanding

of the regulation of bone turnover in normal and disease states.

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