Mechanisms in the skeletal complications of breast cancer

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Abstract

Breast cancer is the most common malignancy in women, with a worldwide prevalence of 1.5 million throughout the industrialized countries. Its mortality rate is second only to lung cancer in the USA and Europe. Its high incidence and prevalence makes it a major public health problem.

Up to one-third of women with early stage breast cancer will eventually succumb to the disease, and most of these will develop bone metastases during the course of the disease. Approximately 70% of patients with breast cancer have bone metastases, with 27% having lung and liver metastases. Hypercalcemia also is very common in advanced breast cancer, manifesting itself in one-third of patients late in the disease. Breast cancer accounts for approximately 25% of the cases of hypercalcemia in cancer.

The major skeletal complications of breast cancer – hypercalcemia and bone metastases – almost certainly share certain mechanisms and these will be discussed.

Hypercalcemia in breast cancer

Hypercalcemia occurs in cancer when the amount of calcium released into the extracellular fluid and blood is so great that it overwhelms the homeostatic controls that operate to keep the calcium within narrow limits in individuals. In humoral hypercalcemia of malignancy (HHM) occurring commonly in many non-breast cancers (Rankin *et al.* 1997), not only is the bone resorption excessive, but also calcium excretion is limited through the renal calcium-conserving action of parathyroid hormone-related protein (PTHrP). The protein was originally purified from a breast cancer (Burtis *et al.* 1987), and PTH-like immunoreactivity (non-parallel to standard PTH) was extracted from a breast cancer of a hypercalcemic patient by Melick *et al.* (1972). Indeed, Paget in 1889 described generalized osteolysis in three women suffering from breast cancer, in whom there was no evidence of bone metastases. Such examples might reflect the occurrence of the HHM syndrome in breast cancer, uncommon though it appears to be. This is so well established as a rare occurrence that if a patient with otherwise uncomplicated breast cancer is found to be hypercalcemic, coexistent primary hyperparathyroidism should be suspected.

Patients with hypercalcemia in breast cancer usually have extensive metastatic disease, with lytic deposits in the skeleton, so that the view was long held that hypercalcemia results from the excessive resorption taking place around

these deposits. If that were correct, the extent of lytic metastases would be expected to correlate with the severity of hypercalcemia. In a clinical study (Ralston *et al.* 1984) in a variety of tumors including breast cancers, no such correlation was found. The same conclusion had been reached by Graham *et al.* (1963), who were unable to find any relationship between the extent of bone metastases and hypercalcemia. By contrast, clinical studies in which renal handling of calcium and phosphate and nephrogenous cyclic AMP were examined, suggested that in up to 60% of hypercalcemic breast cancer patients humoral-mediating factors were operative in the production of hypercalcemia (Percival *et al.* 1985, Isales *et al.* 1987, Gallacher *et al.* 1990). Further evidence that there is at least a humoral component in breast cancer came from the discovery of elevated plasma PTHrP levels in 70% of hypercalcemic patients with bone metastases in breast cancer (Grill *et al.* 1991).

The question of involvement of PTHrP in the hypercalcemia of breast cancer will be discussed along with several other possible contributing factors. Relationship to estrogen status is a recurring question that cannot yet be satisfactorily resolved. Although there are clinical studies indicating that bone metastases are more likely to occur with estrogen-positive tumors, these have not been prospective studies, but often retrospective analyses of selected patient groups (Campbell *et al.* 1981, Stewart *et al.* 1981). It has

long been recognized that treatment with estrogen or anti-estrogens can precipitate serious hypercalcemia in patients with breast cancer (Kennedy *et al.* 1953, 1979, Spooner & Evans 1979).

Mechanisms of metastasis

The metastatic process involves several steps, all of which must be completed successfully for a tumor cell to establish and grow as a secondary deposit. After growth of tumor at the primary site, with accompanying growth of new blood vessels, these vessels provide the cancer cells with an entry route to the circulation because of the high degree of tumor vascularization and increased permeability of these new vessels. Simply getting to the circulation is only part of the process; cells in the circulation need to attach and adhere to extracellular matrix components and to other cells. Integrins are important in this process. These are heterodimeric molecules that bind to the extracellular matrix through Arg-Gly-Asp (RGD) sequences present in matrix proteins. For example, the $\alpha_v\beta_3$ integrin, which is important in osteoclast attachment to bone, is expressed at high levels in breast cancer cells in bone (Liapis *et al.* 1996) and may help breast cancer cells to lodge in bone by its ability to bind the bone proteins vitronectin, bone sialoprotein (BSP) and collagen type-1. This integrin is also expressed in endothelial cells and neutralizing antibodies have been shown to inhibit breast tumor growth and angiogenesis (Brooks *et al.* 1995). Recent studies of primary breast tumors indicate that its expression is elevated in tumors of patients with metastatic disease and that it may have prognostic value in predicting metastatic progression (Gasparini *et al.* 1998).

Certain bone matrix proteins are expressed in breast cancer cells and might contribute to the affinity these cells appear to have for bone. In the invasion process the cancer cells must penetrate the basement membrane and extend through the extracellular matrix. This requires that the normal balance between proteases and their inhibitors be disturbed. The matrix metalloproteinase family of enzymes is central to the invasion process, and the balance between their activation, and their inactivation by the endogenous inhibitors TIMPs (McCawley & Matrisian 2000). Indeed transfection of metastatic breast tumor cells with TIMP-2 reduces bone metastases in an animal model of breast-bone metastasis (Yoneda *et al.* 1997).

Bone metastasis in breast cancer

The predilection of breast cancers to grow as metastases in bone was recognized by Stephen Paget in 1889. Paget's insights were remarkable in that they pointed to the importance of the 'seed' (cancer cells) and their dependence upon the 'soil' (host tissue) in determining the metastatic process. His superb analysis of many hundreds of autopsies

of patients dying with cancer allowed him to develop this hypothesis of metastatic growth that is supported today by our views of the mechanisms of bone metastasis formation. It also encapsulated modern views of the metastatic process in general, i.e. that tumor cells in order to grow in distant organs require special properties that make them suited to those organs (Hart & Fidler 1980, Nicolson 1988).

Paget argued against the view of Virchow (1860) that tumor emboli influenced the tissues in which they lodged to grow like themselves. Rather, he had the idea that certain organs may be predisposed to secondary cancer – 'when a plant goes to seed, its seeds are carried in all directions: but they can only live and grow if they fall on congenial soil.' In examining 650 autopsies carried out on patients with breast cancer he observed the remarkable frequency of secondary growths in bone, especially at the ends of femora and skull. He concluded that 'in cancer of the breast, the bones suffer in a special way, which cannot be explained by any theory of embolism alone. Some bones suffer more than others, the disease has its seats of election'.

In a study of patients with a number of solid tumors by Gutman *et al.* (1936), the patients with malignancy and hypercalcemia (two-thirds had breast cancer) had extensive bone metastases. Only 6 of the 29 breast cancer patients had raised serum calcium levels, leading the authors to postulate that the hypercalcemia was due to rapid localized bone destruction. They reached this conclusion, although they recognized that the hypercalcemia in patients with hyperparathyroidism and multiple myeloma was associated with a general de-mineralization of the skeleton. Further support for the direct tumor osteolysis hypothesis came from a study by Farrow & Woodard (1942) who reported the development of hypercalcemia in patients with breast cancer metastatic to bone that progressed when treated with sex steroids. Milch & Changus (1956) noted the relative paucity of osteoclasts in the region of bone resorption and concluded that the process of malignant bone destruction was most likely due to direct tumor pressure or a physiochemical process due to the presence of tumor cells.

Furthermore, when human breast cancer cells were grown on mouse calvaria *in vitro*, calcium release occurred (Eilon & Mundy 1978), suggesting that direct resorption of bone by tumor cells might take place. However such results are probably explained by release of tissue-degrading enzymes rather than true resorption, with the classical Howship's lacunae in bone resulting from osteoclast activity. There is ample histological evidence that human breast tumor deposits in bone are surrounded by active osteoclasts (Mundy & Martin 1982, Clohisy & Ramnaraine 1998; Fig. 1a), and there is similar evidence from animal models of tumor osteolysis (Clohisy *et al.* 1996, 2000, Clohisy & Ramnaraine 1998) and of metastasis to bone (Lelekakis *et al.* 1999; Fig. 2). Furthermore, the inhibition of osteoclast mediated bone resorption is effective in reducing bone

Figure 1 (a) Haematoxylin and eosin-stained section through bone showing breast cancer cells adjacent to resorbing osteoclasts, as indicated by the arrows. (b) Breast cancer cells staining positive for PTHrP, residing in bone of a normocalcemic patient with metastatic disease. Active osteoclasts, some of which stain positive for PTHrP, can be seen along the bone surface close to tumor cells.

Figure 2 Haematoxylin and eosin-stained section of breast cancer cells residing in bone taken from a mouse orthotopic model of breast cancer metastasis. Active osteoclasts resorbing bone immediately adjacent to tumor cells are indicated by the arrows. (Fig. 2H from Lelekakis *et al.* 1999. Reproduced by kind permission from Kluwer Academic Publishers).

metastatic burden in clinical (Hillner *et al.* 2000, Lipton *et al.* 2000) and experimental (Yoneda *et al.* 1997, 2000) studies.

In the case of cancers establishing in bone, there is little doubt that the single most important property of the cancer

cells is that they promote the formation of active osteoclasts from precursors in the host bone. The remainder of this review will address clinical and experimental data pertaining to the mechanisms by which breast cancers affect the

skeleton, beginning with discussion of several candidate contributing factors.

Clinical studies

Breast cancer is one of several tumors including prostate, thyroid and kidney that displays a remarkable and specific predilection for metastasis to bone. Bone is the commonest site of metastasis from breast cancer and at least 80% of patients who develop metastatic breast cancer will at some time during the course of their disease develop bone metastases (Tubiana-Hulin 1991). Other estimates of the frequency of bone metastases in breast cancer are even higher, with a final incidence of up to 85% (Galasko 1982). Although most patients also have metastases at other sites, approximately 15–17% have no evidence of metastatic disease other than in bone (Hortobagyi *et al.* 1984, Coleman & Rubens 1987). Bone represents the first site of metastasis in >50% of patients who fail systemically (Coleman & Rubens 1987). Survival after the diagnosis of bone metastases is primarily related to the presence of other non-bone metastases. There is wide variation in survival after diagnosis of bone metastases, most series reporting a median survival in the order of 20–30 months (Theriault 1996). This is in contrast to the median survival of patients with soft tissue metastases which is in the order of 3–5 months. Apart from the absence of metastatic disease in other soft tissue sites, a number of factors are known to influence survival after diagnosis of bone metastases. The length of disease-free survival prior to development of bone metastases is an important predictor of outcome, as is pre-menopausal rather than post-menopausal status (Scher & Yagoda 1987). The presence of osteosclerosis as seen on X-ray as the predominant form of metastasis is associated with improved survival. The presence of osteosclerosis has been interpreted as indicating a slower rate of growth (Milch & Changus 1956). Unfortunately >80% of bone metastases from breast cancer noted on plain X-rays are osteolytic in appearance. It is generally accepted that the development of radiological sclerosis in a previously osteolytic lesion indicates tumor regression with healing (Hortobagyi *et al.* 1984). Yamashita *et al.* (1995) have demonstrated prolonged survival in patients developing an osteosclerotic response compared with those patients who have failed to exhibit such a response. Estrogen receptor status of the primary tumor is associated with improved survival but whether this represents an intrinsic property of the tumor or increased likelihood of a favorable response to therapy is unknown. Other properties of the primary tumor that favor development of bone metastases, include lower histological grade, extent of angiogenesis and increased plasminogen activator activity (Sherry *et al.* 1986, Scher & Yagoda 1987).

Although in many cases the bone lesions might be asymptomatic, they nevertheless are a major cause of

morbidity, with bone pain that can be severe and resistant to analgesic control together with susceptibility to fractures that often require surgery. The complication of spinal cord compression following vertebral fracture is an especially difficult one. Hypercalcemia occurs in about 30% of patients with bone metastases, bringing with it distressing symptoms. Major aims of therapeutic development should be to prevent the establishment of metastases in bone and to inhibit the growth of established metastatic deposits.

PTHrP: discovery and properties

The role of PTHrP in the skeletal complications of breast cancer has been the subject of much attention and will be discussed in detail. The discovery of PTHrP as a likely cause of hypercalcemia in many patients with cancer has provided new insights into the pathogenesis of the skeletal complications of malignancy. They show PTHrP to be a previously unrecognized hormone, important in fetal development and in the pathogenesis of hypercalcemia when produced in excess in certain cancers, but otherwise exerting paracrine actions in a number of fetal and adult tissues. The term humoral hypercalcemia of malignancy (HHM) was introduced to describe patients with certain cancers in whom the blood calcium is elevated in the absence of skeletal metastases (Martin & Atkins 1979). The most common cause of this is squamous cell carcinoma of the lung, as well as squamous cell cancers at other sites, including skin, esophagus, and head and neck, and also renal cortical carcinoma, primary liver cancer, pancreatic cancer, bladder carcinoma, and melanoma, and rarely, breast and prostate cancer. In the absence of secondary lesions, removal of the primary tumor leads to resolution of the hypercalcemia (Burtis *et al.* 1990, Grill *et al.* 1991). Tumor factors are secreted that act on the skeleton generally to increase bone resorption, and on the kidney to reduce calcium excretion and increase phosphorus excretion. Nephrogenous cAMP excretion is also increased (Kukreja *et al.* 1980, Stewart *et al.* 1980, Rude *et al.* 1981), and there is often a mild hypokalemic, hypochloremic alkalosis. The similarity of the biochemical features of HHM to those of primary hyperparathyroidism are obvious, and had been recognized as early as the 1930s.

Purification of PTHrP was achieved from a lung cancer cell line (Moseley *et al.* 1987) and a breast cancer (Burtis *et al.* 1987) and with cDNA cloning (Suva *et al.* 1987, Mangin *et al.* 1988) revealing it to be a mature protein of 141 amino acids, in which 8 of the first 13 residues were identical with those in PTH. Alternate splicing of the PTHrP gene yielded proteins of 139, 141 and 173 residues respectively and multiple mRNA species. The amino-terminal similarity in structure to PTH was sufficient to explain the fact that the two proteins are equally effective in acting upon a common PTH/PTHrP receptor. Despite the limited structural

similarity, it has not proven difficult to obtain anti-sera against PTHrP that are free of any detectable cross-reactivity with PTH.

PTHrP is active in promoting bone resorption (Horiuchi *et al.* 1987, Stewart *et al.* 1988, Yates *et al.* 1988, Raisz *et al.* 1990) and, like PTH itself, does so by acting via PTH/PTHrP receptors, first on cells of the osteoblast lineage, with subsequent formation and activation of osteoclasts (Evely *et al.* 1991). Both PTHrP and PTH promote cAMP and phosphorus excretion and reduce calcium excretion. Other actions of PTHrP that reflect those of PTH include the ability to relax vascular and other smooth muscle. This response may reflect more a physiological function of PTHrP rather than of PTH and is consistent with PTHrP production and local action on smooth muscles at various sites (Martin *et al.* 1997).

In addition to the PTH-like actions of PTHrP, there is increasing evidence for other biological activities within the PTHrP molecule, not shared with PTH, and giving rise to the concept that PTHrP is a polypeptide precursor of a number of biological activities, analogous with pro-opiomelanocortin. These include the data suggesting that PTHrP is an oncofetal hormone, circulating in the fetus and acting on the placenta to promote calcium transport from the mother to the fetus (Rodda *et al.* 1988), an effect mediated by a portion of the PTHrP molecule distinct from the PTH-like region. Of great interest is the discovery that PTHrP is localized either in the nucleus or the cytoplasm of cells, and that its location is cell cycle-dependent (Lam *et al.* 1997, 1999a,b). In quiescent cells, nuclear/nucleolar location is evident, with predominant cytoplasmic location and increased production and secretion as cells move towards mitosis (Lam *et al.* 1999a). The nuclear transport of PTHrP is carried out by specific binding to importin β , and phosphorylation of Thr85 of PTHrP by the cyclin-dependent protein kinases, CDK2 and CDC2, favors extrusion of PTHrP from the nucleus (Lam *et al.* 1999b). The nuclear/nucleolar location, its phosphorylation control, cell cycle dependence, and specific nuclear import mechanism all suggest that the protein exerts important function(s) in the nucleus, the nature of which remain to be determined.

The roles of PTHrP in the developing and lactating breast are especially relevant to any function in breast cancer. Demonstration of the prolactin-responsive production of PTHrP by lactating mammary tissue (Thiede & Rodan 1988) led to the finding of large quantities of immunoreactive PTHrP in the milk of several species, including humans (Budayr *et al.* 1989, Grill *et al.* 1992). Suckling-induced rises in urinary phosphate and cyclic AMP in the rat (Yamamoto *et al.* 1992), and a venous–arterial concentration gradient of plasma PTHrP in the goat (Ratcliffe *et al.* 1992) all identify PTHrP as a major product of the activated breast. Its roles are yet to be determined, but are likely paracrine in the

breast, with potential for an endocrine function during lactation.

Such production by the activated breast might be relevant to the place of PTHrP in breast cancer, but its involvement in breast development may be even more important. Mice in which the PTHrP gene is ablated die very soon after birth with predominantly skeletal deformities. Rescue of these mice by directing PTHrP production to cartilage with use of the collagen II promoter has allowed the study of the effect of the PTHrP null phenotype on several other organs. In the case of the breast, the ‘rescued’ PTHrP null mice show failure of early breast ductal development, providing clear evidence of a role for PTHrP in promoting branching morphogenesis (Wysolmerski *et al.* 1995, 1998). With such dramatic expressions of PTHrP involvement both in early breast development and in activation of the mature breast, it is perhaps not surprising that PTHrP emerges as a factor important in breast cancer biology.

Hypercalcemia: role of PTHrP in breast cancer

Expression of PTHrP mRNA and protein have been demonstrated in a large number of tumor types and tumor-derived cell lines. The experiments of Kukreja *et al.* (1990), in which hypercalcemia was treated and prevented by anti-PTHrP antisera in a nude mouse model of HHM, provided strong support for the importance of PTHrP in contributing to the mechanism of hypercalcemia. Subsequent studies have detected PTHrP in >90% of squamous cell carcinomas by either immunohistochemical or *in situ* hybridization techniques, and close to 100% of tumors are positive when the two techniques are combined. Tumor production of PTHrP has also been demonstrated in hematological malignancies, particularly in adult T cell leukemia/lymphoma (Moseley *et al.* 1991), but also in cases of multiple myeloma and in lymphomas of B cell lineage. The regulation of PTHrP gene expression has been extensively studied in adult T cell leukemia/lymphoma, a malignancy associated with human T cell leukemia virus type 1 infection. This malignancy is frequently associated with hypercalcemia.

Further confirmation of the etiological link between elevated levels of PTHrP and hypercalcemia associated with malignancy has been achieved by measurements of circulating levels by radioimmunoassay and immunoradiometric assays, which have documented circulating levels of PTHrP in subjects with malignancy-associated hypercalcemia (Burtis *et al.* 1990, Grill *et al.* 1991). With an N-terminal radioimmunoassay, circulating levels of PTHrP have been found to be elevated in 100% of hypercalcemic patients with solid cancers and no detectable bone metastases (Fig. 3) (Grill *et al.* 1991). Elevated PTHrP levels were found also in 65% of patients with hypercalcemia and breast

Figure 3 PTHrP levels in breast cancer patients with hypercalcemia compared with those in patients with humoral hypercalcemia of malignancy (HHM) associated with non-breast tumors.

carcinoma metastatic to bone. Patients with solid tumors other than breast carcinoma who had hypercalcemia and bone metastases also had elevated PTHrP levels, particularly those with squamous cell carcinoma, of whom 100% had detectable PTHrP immunoreactivity. In normal subjects, no PTHrP assay has convincingly measured PTHrP in the circulation. This is consistent with the view that in normal physiology, PTHrP acts as a cytokine, or local regulator, in the many tissues in which it is produced. This includes skin, bone, uterus, brain and breast. Apart from its role in the fetus, and its appearance in the circulation during lactation, PTHrP only functions as a hormone post-natally when it is produced in sufficient excess by cancers. In the case of breast cancer it seems that PTHrP can exert both paracrine and endocrine roles.

In studying breast cancers removed at surgery, Southby *et al.* (1990) found that two-thirds exhibited positive immunostaining for PTHrP in the cancer cells. A retrospective study of primary breast cancers and metastatic cancers in bone and soft tissue revealed that 85% of metastasis in bone were positive for PTHrP compared with

18% of those at non-bone tissues (Powell *et al.* 1991) (Fig. 1b). This led to the proposal that production of PTHrP as a bone-resorbing factor might be a property of breast cancers that would favor their growth in bone.

This strong evidence invoking PTHrP in breast cancer, and particularly in the bone metastases process, gave rise to informative experimental and clinical studies. Guise *et al.* (1996) used the human breast cancer cell line MDA-MB-231 in a model of tumor-mediated osteolysis in which the cells are injected into the left ventricle of nude mice, and radiology and histology used to examine and quantitate the lytic bone deposits. These typically developed after 3 weeks, with the tumor-bearing mice exhibiting no rise in PTHrP or calcium levels in peripheral blood but elevated PTHrP levels in marrow plasma. Importantly, treatment of tumor-injected animals with a neutralizing monoclonal antibody against PTHrP largely prevented tumor growth and histological evidence of bone invasion. In these studies, tumors growing in bone were marked by prolific osteoclast appearance at the tumor-bone interface, an appearance that was lost with anti-PTHrP treatment.

In a model of spontaneous metastasis of a mouse breast cancer to bone intra-mammary inoculation also results in elevated circulating PTHrP and calcium and osteolytic metastasis (Lelekakis *et al.* 1999). This model is unique in that it represents the entire metastasis pathway from the primary site to bone. Subcloning of the original mouse tumor has given rise to cell lines that either do not metastasize or which metastasize to different sites including lymph nodes, lung and bone. Although all the clones secrete low levels of PTHrP, secretion is greatest in the clone that spreads to bone. Primary tumors derived from all clones stained positive for PTHrP *in vivo*. This model promises to be useful in defining the roles of PTHrP in the early invasive processes as well as in the establishment of bone secondaries.

These experiments have helped in proving the concept that tumor production of a bone-resorbing cytokine, in this case PTHrP, could facilitate cancer establishment in bone. They do not exclude contributions from other cytokines, e.g. interleukins (IL)-1 and -6, tumor necrosis factor (TNF) or of cyclo-oxygenase (COX) products, which could be produced by tumor or host cells in response to the tumor (Fig. 4). Many of these cytokines are able to stimulate PTHrP production and could potentially contribute in this way. However, the

accumulating data showing the frequency with which PTHrP is produced in primary and metastatic breast cancer, focuses attention on its role. The other important feature of these experiments was the prominent part played by osteoclasts in the tumor invasion process. The bisphosphonates, as powerful inhibitors of bone resorption by decreasing osteoclast activity and increasing osteoclast apoptosis, significantly reduce skeletal morbidity in patients with advanced breast cancer (Hortobagyi *et al.* 1996). These drugs have been used with dramatic effect both in the intra-cardiac injection model and the intra-mammary inoculation model to prevent the development of lytic lesions in mice following inoculation with MDA-MB-231 cells (Yoneda *et al.* 1997, Boyce *et al.* 1999).

The importance of bone as the favorable 'soil' for the growth of breast cancer cells as 'seed', is illustrated beautifully by experiments indicating that transforming growth factor (TGF) released from bone matrix during resorption can enhance further tumor invasion in bone by promoting PTHrP production by the cancer cells (Fig. 4). This evidence was obtained by expressing a dominant-negative TGF receptor in the MDA-MB-231 cells, and finding a substantial reduction in tumor establishment and

Figure 4 Potential interactions of breast cancer cells with the bone microenvironment which could lead to increased osteolysis.

growth in bone (Yin *et al.* 1999). Such evidence draws attention to several possible points of intervention in prevention and treatment of bone metastases; the cytokines produced by the cancers, the osteoclast formation and activity, the TGF from bone and its signaling pathways.

Because osteoclast formation is apparently so important in bone metastasis formation and growth, recent discoveries in osteoclast biology provide new points of interest and possibly of therapeutic development. Osteoprotegerin (OPG) is a secreted member of the TNF receptor family that is a product of osteoblastic stromal cells and a powerful inhibitor of osteoclast formation and activity. It achieves this by binding to RANKL, a member of the TNF ligand family that is produced by osteoblastic stromal cells and binds to its receptor, RANK, on hemopoietic cells to induce osteoclast differentiation in the presence of M-CSF (reviewed in Suda *et al.* 1999). When the human breast cancer cell line MCF-7 was engineered to over-express PTHrP, these cells yielded large lytic tumors when administered by intra-cardiac injection to nude mice (Thomas *et al.* 1999). The cells produced OPG and RANK mRNA, but RANKL mRNA could not be detected by reverse transcriptase PCR (RT-PCR). When they were co-cultured with mouse calvarial osteoblasts and hemopoietic cells, osteoclasts were generated without the need for treatment with bone-resorbing agents. Furthermore, expression of RANKL mRNA was enhanced in the co-cultures and OPG mRNA was decreased. Thus the suggestion was that the tumor cells adjacent to host bone cells could promote the formation of osteoclasts by inducing appropriate changes in the production of those molecules that are crucial in the process (Fig. 4).

Data of this type are essentially a proof of concept. Rather than establishing that PTHrP is exclusively involved in such a process it indicates that PTHrP could be a major player, but so too could other tumor cell products capable of promoting osteoclast formation.

Clinical studies: PTHrP and breast cancer

The incidence of 60–70% of positive staining for PTHrP in primary breast cancers has been well confirmed (Southby *et al.* 1990, Bundred *et al.* 1992, Bouizar *et al.* 1993, Kissin *et al.* 1993), and mRNA expression has been shown (Vargas *et al.* 1992, Bouizar *et al.* 1993). Our finding that positive immunostaining for PTHrP-positive tumors was more frequent in bone metastases than in metastases to non-bone sites (Powell *et al.* 1991) resulted from a retrospective analysis of archival tumor specimens. Several subsequent studies which have concluded that PTHrP expression in primary tumors is related to subsequent bone metastasis development (Bundred *et al.* 1992, Bouizar *et al.* 1993, Kissin *et al.* 1993) were either retrospective, had limited numbers of patients or were carried out in selected groups with short follow-up.

The only long-term prospective study of consecutively accrued patients analysis has been carried out on 367 consecutive patients treated by surgery at the one center. With an incidence of 72% of patients with PTHrP-positive breast cancers at the time of operation, the analysis showed that patients with PTHrP-positive tumors had a significantly improved survival ($P=0.001$; Henderson *et al.* 1999). Although this finding appeared to be at odds with the starting hypothesis, which was that expression of PTHrP in primary breast cancers would correlate with subsequent development of bone metastases, it is by no means inconsistent with a role for PTHrP in bone metastasis development. This clinical study suggests that PTHrP negative breast cancers may be relatively enriched in general invasive properties that are needed for metastasis establishment. Upon reaching the bone marrow and exerting these functions, the resulting microenvironment would provide the special properties necessary for invading bone i.e. enhanced production of PTHrP by cancer cells through the actions of local factors, especially TGF, leading to increased osteoclast formation and bone resorption (Fig. 4). The implication from that study, that PTHrP expression in breast cancer cells contributes to a less invasive phenotype, is an intriguing one that is being actively investigated.

Other potential mediators of hypercalcemia in breast cancer

Prostaglandins

Labile products of arachidonic acid metabolism are generated by cancer cells, they are potent inducers of bone resorption in organ culture, and stimulate osteoclast formation in bone marrow culture or in co-cultures of osteoblast/stromal cells with hemopoietic cells. The most potent of the prostanoids in this respect is prostaglandin (PG)E₂ (Martin & Partridge 1980). Their rapidly generated breakdown products, which predominate in the circulation, are much less potent than PGE₂ itself.

Studies in animal models of hypercalcemia in cancer identified PGE₂ as a causal agent. The VX2 carcinoma in the rabbit (Voelkel *et al.* 1975) and the HSDM1 fibrosarcoma in the mouse (Tashjian *et al.* 1972) became very large, and produced high levels of PGE₂. The hypercalcemia in those animals was treated effectively with COX inhibitor drugs. With the Walker carcinosarcoma in the rat, injection of those cells into the aorta led to lytic bone deposits and growths in other organs, as well as hypercalcemia. When aspirin and indomethacin were given to inhibit prostaglandin production, bone deposits and hypercalcemia were prevented, but soft tissue tumor growth was not affected (Powles *et al.* 1973). The findings with prostanoid-related mechanisms were indicative of the general mechanisms in bone invasion, especially for the ways in which they focused attention on the

importance of osteoclasts in its pathogenesis. Galasko (1976) found that within 24 h of injecting VX2 carcinoma cells into rabbit bones, osteoclast formation was evident, with these cells separated from the tumor cells by a small amount of fibrous stroma. As the tumor advanced, osteoclasts were more abundant and caused obvious bone resorption. Furthermore, when tumor cells were injected into thigh muscle or a diffusion chamber containing tumor cells was implanted in muscle, the greatest number of osteoclasts was found in bone close to the tumor cell mass or diffusion chamber. All of these findings pointed to the high probability that diffusible substances from the cancer cells promote osteoclast formation and/or activation, and that this was necessary for tumor establishment and expansion in bone.

The findings in animal models were naturally a stimulus to clinical studies. Co-culture of human breast cancer explants with mouse bone resulted in bone resorption, which was inhibited by indomethacin or aspirin (Dowsett *et al.* 1976a,b, Powles *et al.* 1973, 1976, Atkins *et al.* 1977). It was noted in some such experiments that blockade of prostaglandin synthesis only partially ablated resorption responses, suggesting that other factors could be operative (Greaves *et al.* 1980). Patients whose breast cancers showed no osteolytic activity *in vitro*, were free of bone metastases 3 years after surgery, whereas a significant proportion of those with osteolytic activity had developed metastases (Powles *et al.* 1976). Along the same lines, Bennett *et al.* (1977) found using biological assay methods that the greatest PG-synthesizing activity *in vitro* was associated with breast tumors which ultimately metastasized to bone; however, further studies by this same group failed to confirm this relationship (Bennett *et al.* 1989).

Indomethacin has been disappointing in the treatment of hypercalcemia of malignancy e.g. in breast cancer as studied by Coombes *et al.* (1976), although there have been some anecdotal reports of success in a few patients (Ito *et al.* 1975, Robertson & Baylink 1977). The latter tended to be in patients with very large tumor deposits, possibly with correspondingly greater PGE production. Despite this lack of clinical efficacy the possibility remains that local production of PGs could contribute to the excessive osteoclast formation and resorption around breast cancers in bone in ways that could be significant to the metastasis process. The failure of older drugs should not deter further investigation, especially as it has never been clear how effective such drugs are at getting to sites of active PG synthesis in bone. The availability now of selective COX 2 inhibitors, and ways of looking afresh at the enzymes in the PG synthesis pathway *in situ*, should lead to further studies of the possible local role of PGs.

Cytokines

Several of the cytokines that are powerful stimulators of bone resorption are produced by breast cancers, and thus should

be considered as possible contributors to the bone metastasis process. None have been subjected to such intensive investigation as PTHrP. IL-6 and -11, leukemia inhibitory factor and oncostatin M, which are produced by breast cancer cell lines and human breast cancers (de la Mata *et al.* 1995, Crichton *et al.* 1996, Douglas *et al.* 1997, Lacroix *et al.* 1998, Karczewska *et al.* 2000), all stimulate osteoclast formation and bone resorption but the most effective and intensively studied are IL-6 and IL-11 (Martin *et al.* 1988). Their roles in breast cancer behavior are largely unknown, but production by tumor cells in the bone marrow could produce the same effect as that proposed for PTHrP, that is promotion of osteoclast formation and therefore tumor expansion into bone. For example, there is ample reason to predict that if IL-11 were to be over-expressed in breast cancer cells and inoculated into the circulation of nude mice, enhanced lytic tumor growth would occur. In the case of IL-6, the additional possibility occurs that it could synergize with PTHrP in effects on osteoclast formation, as it has been shown to do in both *in vitro* and *in vivo* experiments (Guise *et al.* 1993). It is noteworthy that many of these cytokines have been shown to regulate PTHrP production (Lacroix *et al.* 1998).

The TNF ligand and receptor super-families have provided the molecules central to the control of osteoclast formation (v supra). The decoy receptor, OPG, which blocks osteoclast formation and activity by binding to RANKL, is effective in preventing and treating hypercalcemia in a murine model of HHM (Capparelli *et al.* 2000). Normally, and most likely in pathological states, OPG is a paracrine regulator, produced by osteoblastic stromal cells and acting locally to limit RANKL-induced osteoclast formation. How it exerts that function when tumor invades bone and whether OPG produced by cancer cells can help in limiting adjacent osteoclast formation remains to be determined. Relevant to this is the question of whether breast cancer cells themselves can produce RANKL, which could provide the cancers with the ability to promote osteoclast formation independently of host osteoblastic cells. The study of Thomas *et al.* (1999), using RT-PCR, revealed that mRNA for OPG is produced by some human breast cancer cell lines and 12 human breast cancers, but no RANKL mRNA was detected in any sample; however, this needs further investigation because it is obvious that there may be important controlling pathways.

Finally, TGF β clearly has a complex role. It is sequestered in the bone matrix and is likely to be abundant at sites of breast tumor lodging in bone marrow, especially during osteoclast-mediated bone destruction at the tumor front. The work of Yin *et al.* (1999) and Guise *et al.* (1997, 2000) using the intra-cardiac inoculation mouse model of breast cancer invasion of bone, has suggested how TGF β in the microenvironment could contribute to tumor invasion of bone by promoting PTHrP production by the invading breast cancer cells (Fig. 4). Another potential contribution by TGF β is suggested by the discovery that it leads to a several-fold

stimulation of osteoclast formation when added to hemopoietic cells in the presence of RANKL and M-CSF (Galvin *et al.* 1999). This is in stark contrast to its known inhibition of osteoclast formation in co-cultures of osteoblasts with hemopoietic cells. Thus in pathological states such as bone metastasis or inflammatory bone disease, availability of TGF β to developing osteoclasts could enhance the process of tumor growth in bone significantly. The signaling pathways used by TGF β in these responses are not yet elucidated but recent evidence suggests that SMAD signaling mediates the PTHrP response to TGF in breast cancer cells (Yin *et al.* 1999).

Bone proteins and matrix metalloproteinases

Although much attention in the study of bone metastases is directed at promotion of bone resorption, there are likely to be other specific properties that enhance the ability of breast cancers to grow in bone. First is the need to be able to attach to, and interact with, bone extracellular matrix. It is increasingly recognized that breast cancers produce certain proteins that are typical of the osteoblast, and this may be a relevant property. Second, the matrix metalloproteinases (MMPs) comprise a large family (at least 20 members) of endopeptidases with substrate specificities for several collagens and non-collagenous proteins of the extracellular matrix (Benaud *et al.* 1998). They are involved in the tissue remodeling that occurs in bone remodeling, normal breast development and wound healing, as well as in tumor invasion and metastasis (Rudolph-Owen & Matrisian 1998).

The production by breast cancers of bone proteins is quite a striking property of tumor cells. BSP is commonly expressed in breast cancers (Bellahcene *et al.* 1994, Gillespie *et al.* 1997), and some evidence suggests that this property relates to the development of bone metastases (Bellahcene *et al.* 1996a) and poor patient survival (Bellahcene *et al.* 1996b). This protein possesses an integrin-binding RGD domain that could promote interactions between breast cancer cells and bone matrix. Evidence in favor of this was obtained in studies of human breast cancer cell adhesion and migration in which promotion by BSP appeared to be through $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors (Sung *et al.* 1998).

Osteopontin is another bone protein related to BSP, also possessing an RGD sequence and able to bind to bone mineral. It was found to be expressed in 100% of a small series of primary breast cancer (Gillespie *et al.* 1997). It too could contribute to breast cancer attachment to bone via the vitronectin receptor. Osteonectin is associated with morphogenesis and tissue remodeling, influencing several cell functions including spreading and adhesion. Also, it is produced in breast cancers (Graham *et al.* 1997) and has been shown to induce MMP 2 activation in human breast cancer cell lines (Gilles *et al.* 1998). These proteins as products of breast cancer cells could combine to confer upon the cells

special properties that, when combined with their ability to promote bone resorption, equip them to establish as metastatic growths in bone.

The fact that MMPs are involved in tissue invasion by cancers, including those of breast, is accepted. In the case of invasion and metastasis in bone, extracellular matrix tissue remodeling is necessary as well as the specific osteoclast-mediated bone invasion. In that sense MMP involvement is likely, but there is little direct information at present.

Implications for treatment and prevention

The importance of osteoclast formation and activity in the development of skeletal complications of breast cancer has led to bone resorption inhibitors having a central place in treatment. The most widely used are the bisphosphonates, analogs of pyrophosphate that concentrate in bone and are, to date, the most effective inhibitors of resorption. Treatment with parenteral bisphosphonates, for example pamidronate, given intravenously every 3–4 weeks, is associated with reduction in skeletal complications of breast cancer, including pathological fractures and their sequelae, treatment requirements (surgery, viradiation), and hypercalcemia, (reviewed in Hillner *et al.* 2000, Hortobagyi *et al.* 1996, 1998, Berenson & Lipton 1999, Lipton *et al.* 2000). However, there is no evidence that such reduction in morbidity leads to prolonged patient survival, nor has there been any prospective clinical study that asks whether bisphosphonates can provide benefit when used in an adjuvant setting in women with breast cancer which has not yet metastasized to bone. Such studies will be important, whether carried out with bisphosphonates, with newer inhibitors of bone resorption as they are developed (Rodan & Martin 2000), or in combination with drugs targeting other aspects of the bone metastasis process, such as the matrix metalloproteinases.

Summary

Hypercalcemia in breast cancer is generally associated with the production of PTHrP and bone metastasis. Whereas PTHrP released into the circulation can cause hypercalcemia of malignancy, and regulation of tumor PTHrP by factors in the bone environment can promote bone metastasis and osteolysis, other cytokines and mediators of bone resorption are also likely to be important. They may be produced by the tumor itself, residing in bone and influenced by local bone factors, or by bone or cells of the immune system in response to the tumor. Successful treatment regimes for breast cancer hypercalcemia and bone metastases will only be realized if all these contributing mechanisms and their interactions are fully understood.

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