Hypercalcaemia of malignancy and basic research on mechanisms responsible for osteolytic and osteoblastic metastasis to bone

G A Clines and T A Guise

Division of Endocrinology and Metabolism, Department of Medicine, University of Virginia, PO Box 801419, Charlottesville, Virginia 22908-1419, USA

(Requests for offprints should be addressed to T A Guise; Email: tag4n@virginia.edu)

Abstract

Calcium homeostasis is a tightly regulated process involving the co-ordinated efforts of the skeleton, kidney, parathyroid glands and intestine. Neoplasms can alter this homeostasis indirectly through the production of endocrine factors resulting in humoral hypercalcaemia of malignancy. Relatively common with breast and lung cancer, this paraneoplastic condition is most often due to tumour production of parathyroid hormone-related protein and ensuing increased osteoclastic bone resorption. Although control of hypercalcaemia is generally successful, the development of this complication is associated with a poor prognosis. The metastasis of tumour cells to bone represents another skeletal complication of malignancy. As explained in the 'seed and soil' hypothesis, bone represents a fertile ground for cancer cells to flourish. The molecular mechanisms of this mutually beneficial relationship between bone and cancer cells are beginning to be understood. In the case of osteolytic bone disease, tumour-produced parathyroid hormone-related protein stimulates osteoclasts that in turn secrete tumour-activating transforming growth factor- β that further stimulates local cancer cells. This 'vicious cycle' of bone metastases represents reciprocal bone/cancer cellular signals that likely modulate osteoblastic bone metastatic lesions as well. The development of targeted therapies to either block initial cancer cell chemotaxis, invasion and adhesion or to break the 'vicious cycle' is dependent on a more complete understanding of bone metastases. Although bisphosphonates delay progression of skeletal metastases, it is clear that more effective therapies are needed. Cancer-associated bone morbidity remains a major public health problem, and to improve therapy and prevention it is important to understand the pathophysiology of the effects of cancer on bone. This review will detail scientific advances regarding this area.

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Introduction

Cancer adversely affects bone and mineral metabolism through a broad spectrum of mechanisms. While focal osteolysis at sites of metastases is the most common skeletal manifestation of cancer, systemic effects such as hypercalcaemia or diffuse osteopenia are common. As early as 1889, Stephen Paget recognized the diversity of effects, stating that 'in a cancer of the breast the bones suffer in a special way, which cannot be explained by any theory of embolism alone ... the

same thing is seen much more clearly in those cases of cancer of the thyroid body where secondary deposition occurs in bones with astonishing frequency.' He further observed 'A general degradation of the bones sometimes occurs in carcinoma of the breast, yet without any distinct deposition of cancer in them.' These were prescient observations, as it is now recognized that cancer affects bone both by direct metastatic invasion and through systemic humoral mechanisms.

Primary bone tumours make up a small minority of bone-related malignancy. This review will therefore

focus on the mechanisms by which extra-skeletal cancers affect bone: (1) directly through secondary spread of tumour to bone both by local extension and by distant metastasis and (2) indirectly through elaboration of factors that act to disrupt normal calcium homeostasis at the level of the kidney, bone, or both.

Each of the three most common human neoplasms — breast, prostate and lung — is strongly associated with skeletal morbidity. In the USA alone, it is estimated that in 2004 there would be 217 440 new cases of breast cancer and 230 110 new cases of prostate cancer resulting in 40 580 and 29 900 deaths respectively. At the same time, the combined number of lung cancers for males and females was estimated to be 173 770 with 160 440 deaths (Jemal *et al.* 2004). The majority of patients dying from these cancers will have bony involvement either through metastatic spread or as a result of systemic tumour-produced factors. Clearly, cancer-associated bone morbidity remains a major public health problem. To improve therapy and prevention it is important to understand the pathophysiology of the effects of cancer on bone. This review will attempt to detail scientific advances regarding this area.

Normal bone remodelling and calcium homeostasis

Bone remodelling

A thorough understanding of the normal mechanisms of bone and calcium homeostasis is necessary to appreciate how perturbations in these mechanisms cause hypercalcaemia of malignancy, as well as osteolytic and osteoblastic lesions. Unlike any other cancer-affected tissue, bone is being constantly remodelled as a result of the co-ordinated actions of bone-forming osteoblasts and bone-resorbing osteoclasts. This remodelling is highly influenced by both circulating systemic hormones and local bone-derived growth factors. Bone is made up of two physically and biologically distinctive structures: the hard, mineralised matrix of cortical bone and the more metabolically active cancellous or trabecular bone. Cortical bone makes up 85% of the total bone mass and is found primarily in the long bones of the appendicular skeletal. Trabecular bone, which makes up the remaining 15%, is most predominant in the vertebral bodies and pelvis. Within trabecular bone is the multicellular bone marrow. This space harbours stromal and haematopoietic stem cells with the capacity to differentiate and form osteoblasts and osteoclasts respectively. These

cells secrete cytokines and growth factors that have either direct actions on neighbouring cells or become immobilised within the mineralised matrix (Yoneda 1996). Thus, the mineralised matrix is a rich source of insulin-like growth factors (IGFs)-I and II, transforming growth factor- β (TGF β), platelet-derived growth factors (PDGFs) and bone morphogenetic proteins (BMPs) (Hauschka *et al.* 1986, Mohan & Baylink 1991). These growth factors, however, are unable to bind to their receptors until released from the matrix as a result of osteoclastic bone resorption (Pfeilschifter & Mundy 1987), a component of the bone remodelling process.

Recent insights into the interaction of marrow stromal cells of osteoblastic lineage with osteoclasts have provided a better understanding of the mechanisms regulating osteoclast activation and bone resorption. Osteoclastogenesis is a stromal cell and osteoblast-dependent process that is mediated by the receptor activator of nuclear factor κ B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) system. Stromal and osteoblast cell expression of the membrane-bound RANKL (Lacey *et al.* 1998) is increased by a number of stimuli, most notable parathyroid hormone (PTH). The receptor for this ligand, RANK, is a high-affinity receptor of osteoclast precursors, as well as T and B cells, fibroblasts and dendritic cells (Anderson *et al.* 1997). When unopposed, RANKL binds RANK and induces osteoclast formation in the presence of Macrophage (M)-colony stimulating factor (M-CSF) (Lacey *et al.* 2000). OPG, a member of the tumour necrosis factor (TNF) receptor superfamily, is a secreted RANKL decoy receptor of osteoblastic-lineage cells (Simonet *et al.* 1997, Yasuda *et al.* 1998). OPG achieves its effect on osteoclasts indirectly by binding to, and blocking the effect of RANKL (Lacey *et al.* 1998). It therefore appears that OPG competes with RANK for association with RANKL and that the ratio of OPG/RANKL is a determinate of osteoclast development. The expression of OPG and RANKL is modulated by a variety of osteotrophic factors, as will be discussed in subsequent sections.

Calcium homeostasis

Serum calcium concentration is highly regulated by a complex system of calcitropic hormones with actions at the levels of bone, kidney and gut (Fig. 1). PTH and the biologically active form of vitamin D (calcitriol or 1,25-dihydroxyvitamin D (1,25-(OH) $_2$ D $_3$)) act on these organs to keep blood ionized calcium levels remarkably stable. Serum calcium concentration is

Figure 1 Calcium homeostasis for a normal adult in zero calcium balance. The numbers are estimates of the amount of calcium exchanged between the extracellular fluid and gut, kidney and bone each day. The exchange system between bone fluid and the extracellular fluid is not taken into account. (Adapted from Mundy (1995)). d, day.

maintained within a very narrow range by the interplay of these two calcitropic hormones with their target tissues of bone, kidney and gut. Under normal conditions, the net exchange of calcium from extracellular fluid to these organs remains at zero balance. PTH and vitamin D are the most physiologically relevant calcitropic hormones in humans, while calcitonin plays a much lesser role. Normal calcium homeostasis has been extensively reviewed (Mundy & Guise 1999). Since most skeletal manifestations of malignancy involve pathways intrinsic to PTH or vitamin D action, these will be further reviewed here.

PTH

PTH is an 84 amino acid polypeptide that is secreted by the chief cells of the parathyroid glands. Regulation of secretion is highly dependent on the ionized calcium concentration in the extracellular fluid. Serum PTH concentration decreases as the ionized calcium concentration increases, representing a simple negative feedback loop. In 1993, the calcium-sensing receptor (CaR) that mediates this feedback loop was cloned from bovine parathyroid cells (Brown *et al.* 1993). CaR is a member of the G protein-coupled receptor family. Activation of this receptor ultimately leads to a

down-regulation of PTH at the post-transcriptional level (Moallem *et al.* 1998, Moz *et al.* 2002). In addition to ionized calcium, calcitriol is a potent inhibitor of PTH secretion (Silver *et al.* 1986, Okazaki *et al.* 1988) and hyperphosphataemia increases PTH secretion (Moallem *et al.* 1998, Tatsumi *et al.* 1998).

The biological actions of PTH include: (1) stimulation of osteoclastic bone resorption, (2) stimulation of calcium reabsorption and inhibition of phosphate reabsorption from renal tubules and (3) stimulation of renal 1α -hydroxylase resulting in production of $1,25-(\text{OH})_2\text{D}_3$, which increases intestinal absorption of calcium and phosphate. These actions result in an increased serum calcium and increased urinary phosphate excretion. PTH actions are mediated through binding of the amino terminus of the PTH molecule to the PTH receptor, a member of the family of G protein-coupled receptors that contain seven transmembrane-spanning domains (Juppner *et al.* 1991). The ligand-bound PTH receptor activates adenylate cyclase, through the activation of G protein $\text{G}\alpha_s$, producing cAMP while activating protein kinase A. Although the majority of the PTH signal appears to be transduced through this pathway, the phospholipase C/ protein kinase C system also contributes to PTH signal transduction (Mahon *et al.* 2002, Swarthout *et al.* 2002).

Table 2. Clinical features of hypercalcaemia (Mundy & Martin 1982)

Neurologic and psychiatric	Lethargy, drowsiness, confusion, disorientation, disturbed sleep, nightmares, irritability, depression, hypotonia, decreased deep tendon reflexes, stupor, coma
Gastrointestinal	Anorexia, vomiting, constipation, peptic ulceration, acute pancreatitis
Cardiovascular	Arrhythmias, synergism with digoxin, hypertension
Renal	Polyuria, polydipsia, hypercalciuria, nephrocalcinosis, impaired glomerular filtration

on the laboratory. Ionised calcium is a more precise measure of calcium concentration, the normal plasma concentrations ranging from 1.12 to 1.23 mmol/l (Favus 2003). However, measurement of total serum calcium, when adjusted for serum albumin, is sufficient for most situations. With the advent of automated biochemical testing, hypercalcaemia is now recognized to be more common than once realized. By far, the most common causes of hypercalcaemia are primary hyperparathyroidism (1°HPT) and malignancy.

The clinical features of hypercalcaemia are featured in Table 2. Neuromuscular, gastrointestinal and renal manifestations are most common. Optimal neurological function is dependent on extracellular calcium level and symptoms can range from subtle neuropsychiatric manifestations, such as irritability and depression, to muscle weakness, delirium and even coma (LeBoff & Mikulec 2003). Non-specific gastrointestinal symptoms of anorexia, constipation, nausea and vomiting may predominate. Hypercalcaemia causes an impaired ability of the distal nephron to concentrate urine and is an important part of the differential diagnosis for nephrogenic diabetes insipidus. Symptoms may vary in individual patients and are related both to the absolute concentration of serum calcium and to the rate of rise in serum calcium. Severe hypercalcaemia is likely the result of a vicious cycle. The hypercalcaemic effects of anorexia, nausea, vomiting and impaired renal concentrating ability lead to dehydration and, subsequently, altered mental status. This, in turn, may promote immobilization and lead to worsening hypercalcaemia. In addition to the symptoms of hypercalcaemia, clinical features of hypercalcaemia of malignancy include signs and symptoms of the underlying cancer. Generally, the cancer is well advanced when hypercalcaemia occurs, and the prognosis is poor. Survival beyond 6 months is uncommon (Ralston *et al.* 1990).

Table 3. Malignancies associated with hypercalcaemia (Mundy & Martin 1982)

Malignancy	Frequency (%)
Lung	35
Breast	25
Haematologic	14
Head and neck	6
Renal	3
Prostate	3
Unknown primary	7
Others	7

Hypercalcaemia of malignancy

Malignancy is the most common cause of hypercalcaemia in the hospitalized patient, and malignancy-associated hypercalcaemia is one of the more common paraneoplastic syndromes. Up to 30% of patients with cancer may develop hypercalcaemia during the course of their disease (Grill & Martin 2000). The relative frequencies of malignancies associated with hypercalcaemia are listed in Table 3 (Mundy & Martin 1982). Hypercalcaemia occurring in the setting of malignancy may be due to (1) humoral factors secreted by tumours that act systemically on target organs of bone, kidney and intestine to disrupt normal calcium homeostasis, (2) local factors secreted by tumours in bone, either metastatic or haematological, which directly stimulate osteoclastic bone resorption and (3) coexisting 1°HPT.

The first two of these situations should be viewed as opposite ends of a continual spectrum, rather than as completely unrelated pathologies. Clearly the pathophysiology of hypercalcaemia in patients with solid tumours and no bone metastases at one end of the spectrum, and myeloma associated with extensive local bone destruction adjacent to the tumour cells at the other, is very different. However, in between these extremes are hypercalcaemic patients with squamous cell carcinomas in which hypercalcaemia may occur with some, but not extensive, osteolytic bone metastases, and hypercalcaemic patients with advanced breast carcinoma in whom hypercalcaemia almost never occurs in the absence of extensive osteolytic bone destruction. Separating hypercalcaemia into sub-categories based on the assumption that the underlying mechanisms are distinct is not entirely satisfactory. This is because the mediators may be identical, except that in one situation it is a local mediator while in another it is a humoral mediator. Additionally, if the tumour burden in bone is great, local tumour-produced mediators of bone resorption may be

present in sufficient quantities to have systemic effects. Although this review will discuss the effects of cancer on bone organised into such categories, it is important to understand that a significant portion of cancer patients will fall into the middle of this spectrum, having both humoral and local effects of tumour on bone.

Humoral mediators of hypercalcaemia of malignancy

Parathyroid hormone-related protein (PTHrP)

Hypercalcaemia has been associated with malignancy since the development of serum calcium assays in the 1920s. Only in 1941, however, did Fuller Albright advance the hypothesis that PTH is ectopically produced by certain tumour types. Over the subsequent five decades investigators determined that PTH was not, in fact, the cause of humoral hypercalcaemia of malignancy (HHM), but that a 'PTH-like factor' was responsible for most cases. In 1987, this PTHrP was purified from human lung cancer (Moseley *et al.* 1987), breast cancer (Burtis *et al.* 1990) and renal cell carcinoma (Strewler *et al.* 1987) simultaneously by several independent groups and cloned shortly thereafter (Suva *et al.* 1987).

PTHrP shares 70% sequence homology with PTH over the first 13 amino acids at the N-terminus. Beyond the initial amino-terminal sequence the protein is unique and shows no further sequence homology with PTH. The cloned PTHrP protein is larger than PTH and three distinct isoforms of 139, 141 and 173 amino acids have been described. Similarly, the human PTHrP gene is much larger and more complex than the PTH gene, spanning 15 kb of genomic DNA and having nine exons and three promoters. Despite these differences, both PTH and PTHrP bind to a common PTH/PTHrP receptor (Abou-Samra *et al.* 1992) and share similar biological activities (Horiuchi *et al.* 1987).

PTHrP has been detected in a variety of tumour types as well as in normal tissue (Danks *et al.* 1989, Asa *et al.* 1990). The widespread expression of PTHrP in normal tissue was the first evidence that the hormone had a role in normal physiology. In addition to the PTH-like effects, emerging work testifies to the fact that PTHrP plays a role in (1) the regulation of cartilage differentiation and bone formation (Minina *et al.* 2001), (2) the growth and differentiation of skin (Wysolmerski *et al.* 1994b), mammary gland (Wysolmerski *et al.* 1998) and teeth (Philbrick *et al.* 1998), (3) cardiovascular function (Schluter & Piper 1998), (4) transepithelial calcium transport in mammary epithelia and placenta (Wysolmerski *et al.* 1994a,

Kovacs *et al.* 1996), (5) relaxation of smooth muscle in uterus, bladder, arteries and ileum (Thiede *et al.* 1990, Yamamoto *et al.* 1992, Botella *et al.* 1994, Pirola *et al.* 1994) and (6) host immune function (Funk *et al.* 1994, 1995). Normal subjects do not have detectable circulating levels of PTHrP, suggesting that in normal physiology PTHrP acts as a local regulator or cytokine in the tissues where it is produced.

Approximately 80% of hypercalcaemic patients with solid tumours have detectable or increased plasma concentrations of PTHrP (Burtis *et al.* 1990). In addition to the diverse normal physiological functions of PTHrP, it has a multifunctional role in cancer as well. Such identified functions include (1) mediating hypercalcaemia, (2) aiding in the development and progression of osteolytic bone metastasis, as will be discussed in subsequent sections, (3) regulating growth of cancer cells (Luparello *et al.* 1993, 1995, Li *et al.* 1996) and (4) acting as a cell survival factor (Chen *et al.* 2002a). The first identified consequence of PTHrP in cancer was the HHM syndrome.

HHM vs 1°HPT. Despite similarities between the syndromes of HHM and 1°HPT and the similar biological activities of PTHrP and PTH respectively, unexplained differences between these syndromes exist. First, patients with PTHrP-mediated HHM have low 1,25-(OH)₂D₃ levels compared with patients with 1°HPT, even though both hormones stimulate 1α-hydroxylase activity (Schilling *et al.* 1993). Moreover, infusion of PTH or PTHrP in healthy volunteers over a 48-h period increased plasma 1,25-(OH)₂D₃ levels; however, PTH was more effective than PTHrP (Horwitz *et al.* 2003). Secondly, while both syndromes have marked increases in osteoclastic bone resorption, many patients with HHM do not have the normally coupled increase in osteoblastic activity that those with 1°HPT experience. Studies using either serum markers of bone turnover (Nakayama *et al.* 1996) or quantitative bone histomorphometry (Stewart *et al.* 1982) have demonstrated this uncoupling of bone resorption from bone formation. Finally, unlike the metabolic acidosis seen in patients with 1°HPT, patients with HHM often have a metabolic alkalosis with a low plasma chloride and high plasma bicarbonate concentration. Although many explanations have been postulated for the discrepancies between HHM and 1°HPT, such as the pulsatile secretion of PTH and the apparent continuous secretion of PTHrP and biologically active PTHrP fragment (Plawner *et al.* 1995) and suppression of bone formation and 1α-hydroxylase activity by other tumour-associated

factors, the reasons for these differences have not been adequately elucidated.

PTHrP in hypercalcaemia associated with breast cancer. The role of PTHrP in breast cancer-associated hypercalcaemia deserves special consideration. Breast cancer predominantly affects bone through metastatic mechanisms, typically with lytic deposits in the skeleton. Approximately 10% of women with breast cancer will have hypercalcaemia as a complication at some point in the disease. The association of hypercalcaemia with extensive osteolytic lesions in breast cancer is so strong that the presentation of hypercalcaemia without bone metastases should suggest the presence of coexistent 1° HPT (Fierabracci *et al.* 2001). Based on this association, it was long held that breast cancer-associated hypercalcaemia results from excessive reabsorption of bone around tumour deposits. However, clinical studies (Ralston *et al.* 1990) failed to demonstrate any relationship between the extent of bone metastasis and serum calcium levels. Conversely, studies exploring the potential role for a humoral mediator of hypercalcaemia in breast cancer clearly demonstrated altered renal handling of calcium and phosphate, as well as increased nephrogenous cAMP, suggesting that 10–60% of hypercalcaemic patients with breast cancer had a circulating factor with PTH-like properties. The identification of PTHrP as this factor is hardly surprising when it is recalled that PTHrP appears to play an important role in normal breast physiology. In the case of breast cancer, PTHrP appears to have both paracrine and endocrine actions.

PTHrP was detected by immunohistochemical staining in 60% of 102 invasive breast tumours removed from normocalcaemic women, but not in normal breast tissue (Southby *et al.* 1990). At least four other studies have confirmed these percentages, and one of these has demonstrated immunoreactive PTHrP within the cytoplasm of lobular and ductal epithelial cells in normal and fibrocystic breast tissues (Bundred *et al.* 1991, 1992, Liapis *et al.* 1993, Kohno *et al.* 1994). Furthermore, 65–92% of hypercalcaemic breast cancer patients (with and without bone metastasis) had detectable plasma PTHrP concentration by radioimmunoassay similar to those documented in patients with HHM due to non-breast tumours (Bundred *et al.* 1991, Grill *et al.* 1991). PTHrP is an important mediator of hypercalcaemia in breast cancer. It also plays a significant role in the pathophysiology of breast cancer metastasis to bone, as evidenced by clinical studies indicating that PTHrP expression by primary breast cancer is more commonly associated with the development of bone metastasis and hypercalcaemia

(Bundred *et al.* 1992). This topic will be discussed in a later section.

PTHrP in hypercalcaemia associated with haematologic malignancies. Hypercalcaemia associated with haematologic malignancies results both from systemic effects of tumour-produced factors such as $1,25-(\text{OH})_2\text{D}_3$ (discussed below), and from secretion of local bone-active cytokines, such as interleukin (IL)-6, IL-1 and lymphotoxin or $\text{TNF}\beta$, from tumour in bone. PTHrP has been demonstrated to be an important pathogenic factor in the development of hypercalcaemia in some patients with haematologic malignancies. In a clinical study of 76 patients with various haematological malignancies, 50% of the 14 hypercalcaemic patients had significant increases in plasma PTHrP concentrations (Kremer *et al.* 1996). Of these, five had non-Hodgkin's lymphoma, one had Hodgkin's disease and one had multiple myeloma. The serum $1,25-(\text{OH})_2\text{D}_3$ concentrations, when measured, were low in the hypercalcaemic non-Hodgkin's lymphoma patients who had increased plasma PTHrP concentrations. Also of interest in this study is the fact that several normocalcaemic patients with non-Hodgkin's lymphoma, Hodgkin's lymphoma, multiple myeloma and Waldenstrom's macroglobulinaemia had increased plasma PTHrP concentrations as measured by an amino-terminal PTHrP assay. Using a sensitive two-site immunoradiometric assay, other investigators have noted increased plasma PTHrP concentrations in patients with adult T cell leukaemia and B cell lymphoma (Ikeda *et al.* 1994). Finally, in separate studies, circulating concentrations of PTHrP, comparable with those in HHM, were present in two of four hypercalcaemic patients with non-Hodgkin's lymphoma, in three of nine with myeloma and in a patient with myeloid blast crisis of chronic myeloid leukaemia (Seymour *et al.* 1993, Firkin *et al.* 1996). Thus, the humoral mediators in the hypercalcaemia associated with haematological malignancies include both $1,25-(\text{OH})_2\text{D}_3$ and PTHrP.

1,25-(OH)₂D₃

Under normal physiological conditions, serum calcium concentration and levels of $1,25-(\text{OH})_2\text{D}_3$ are inversely related. In the setting of hypercalcaemia, serum $1,25-(\text{OH})_2\text{D}_3$ concentrations are suppressed unless there is persistent stimulation of 1α -hydroxylase activity from an autonomous source of PTH, such as with 1° HPT. Outside of this situation, lack of $1,25-(\text{OH})_2\text{D}_3$ suppression indicates abnormal regulation of $1,25-(\text{OH})_2\text{D}_3$ synthesis and further suggests extra-renal production such as that observed with granulomatous

disease. In granulomatous disease, activated macrophages within the granuloma synthesise $1,25\text{-(OH)}_2\text{D}_3$ (Adams *et al.* 1983, Gkonos *et al.* 1984, Mason *et al.* 1984). Similarly, a major mediator of hypercalcaemia in Hodgkin's disease, non-Hodgkin's lymphoma and other haematological malignancies appears to be an extra-renal production of $1,25\text{-(OH)}_2\text{D}_3$ (Seymour *et al.* 1994). In this scenario, hypercalcaemic patients have increased plasma $1,25\text{-(OH)}_2\text{D}_3$ concentrations with low or normal plasma PTH and urinary cAMP concentrations (Rosenthal *et al.* 1985), without the presence of bone involvement. Affected patients have also been shown to have increased fasting urinary calcium excretion (Rosenthal *et al.* 1985), as well as increased intestinal calcium (^{47}Ca) absorption (Breslau *et al.* 1984). Increased $1,25\text{-(OH)}_2\text{D}_3$ concentrations were noted in 12 of 22 hypercalcaemic patients with non-Hodgkin's lymphoma. In addition, 71% of 22 normocalcaemic patients with non-Hodgkin's lymphoma were hypercalciuric, and 18% had increased serum $1,25\text{-(OH)}_2\text{D}_3$ concentrations. These findings led the investigators to conclude that dysregulated $1,25\text{-(OH)}_2\text{D}_3$ production was common in patients with diffuse large cell lymphoma (Seymour *et al.* 1994). The low serum PTH and urinary cAMP concentrations indicated that neither PTH nor PTHrP mediated the hypercalcaemia in this setting. Prostaglandins, when measured, have been low, and selected patients had no calcium-lowering effect from indomethacin therapy (Firkin *et al.* 1996).

Thus, the mechanisms responsible for hypercalcaemia in this setting appear to be multifactorial and include $1,25\text{-(OH)}_2\text{D}_3$ -mediated increases in intestinal absorption of calcium and osteoclastic bone resorption. Additionally, many of the reported patients had altered renal function, a finding that suggests that impaired renal calcium clearance may also be contributing to the hypercalcaemia in certain patients. It is likely that the lymphoma tissue itself hydroxylates $25\text{-(OH)}\text{D}_3$ to the active $1,25\text{-(OH)}_2\text{D}_3$ similar to the situation in hypercalcaemia associated with granulomatous disease. One α -hydroxylase activity has been demonstrated in human T cell lymphotropic virus type-I-transformed lymphocytes as well as in other extra-renal tissues (Fetchick *et al.* 1986, Zehnder *et al.* 2001). None of the reported patients with $1,25\text{-(OH)}_2\text{D}_3$ -mediated hypercalcaemia had concomitant granulomatous disease, and hypercalcaemia often improved with medical or surgical therapy that resulted in a decrease in serum $1,25\text{-(OH)}_2\text{D}_3$ concentration. Recurrence of hypercalcaemia and increased plasma $1,25\text{-(OH)}_2\text{D}_3$ concentrations have been documented with recurrence of disease (Mercier *et al.* 1988).

PTH

For many years after Fuller Albright's observations in 1941 malignancy-associated hypercalcaemia was attributed to ectopic tumour-produced PTH. It is now clear that PTHrP is responsible in the great majority of cases of HHM. There have been rare cases, however, of authentic tumour-produced PTH causing hypercalcaemia. Specifically, ectopic PTH production has been documented in a small cell carcinoma of the lung (Yoshimoto *et al.* 1989), a squamous cell carcinoma of the lung (Nielsen *et al.* 1996), an ovarian cancer (Nussbaum *et al.* 1990), a widely metastatic primitive neuroectodermal tumour (Strewler *et al.* 1993), a papillary adenocarcinoma of the thyroid (Iguchi *et al.* 1998) and a thymoma (Rizzoli *et al.* 1994). Molecular analysis of the ovarian carcinoma revealed both DNA amplification and rearrangement in the upstream regulatory region of the PTH gene. Interestingly, the primitive neuroectodermal tumour produced both PTH and PTHrP that resulted in severe hypercalcaemia. These reported patients did not have coexisting 1°HPT since the parathyroid glands were normal at the time of neck exploration or at autopsy in all cases. However, the fact remains that ectopic production of PTH is a rare event, and it is clearly documented that most patients with malignancy-associated hypercalcaemia have suppressed plasma PTH concentrations (Stewart *et al.* 1980). It should be emphasised that the most likely cause of hypercalcaemia in the setting of malignancy that is associated with a normal or increased serum PTH concentration is co-existing hyperparathyroidism.

Other tumour-associated factors

There is accumulating evidence that solid tumours may produce other humoral factors, either alone or in combination with PTHrP, that have the capacity to stimulate osteoclastic bone resorption and cause hypercalcaemia. These factors include IL-1, IL-6, $\text{TGF}\alpha$, TNF and granulocyte CSF (G-CSF). Administration of IL-1 injections to mice caused a mild hypercalcaemia (Sabatini *et al.* 1988, Boyce *et al.* 1989), which has been effectively blocked by an IL-1 receptor antagonist (Guise *et al.* 1993). Mice bearing Chinese hamster ovarian (CHO) tumours transfected with the cDNA for IL-6 developed mild hypercalcaemia (Black *et al.* 1991), as did mice bearing a renal carcinoma that co-secreted IL-6 and PTHrP (Weissglas *et al.* 1995). High serum levels of IL-6 in patients with HHM have also been reported (Barhoum *et al.* 1999, Ueno *et al.* 2000).

Human $\text{TGF}\alpha$ and $\text{TNF}\alpha$ stimulated osteoclastic bone resorption *in vitro* and resulted in hypercalcaemia

in vivo (Ibbotson *et al.* 1985, Bertolini *et al.* 1986, Yates *et al.* 1992). TNF α also caused hypercalciuria, without an increase in nephrogenous cAMP, and increased osteoclastic bone resorption *in vivo* in a mouse model (Johnson *et al.* 1989). In addition, as noted in the previous section, some of these factors have been shown to modulate the end-organ effects of PTHrP on bone and kidney. In some instances, factors such as TGF α , IL-1, IL-6 and TNF enhanced the hypercalcaemic effects of PTHrP. The ability of IL-6 to enhance PTHrP-mediated hypercalcaemia appeared to be due to increased production of the early osteoclast precursors by IL-6 (as measured by granulocyte macrophage colony-forming units), in combination with increased production of the more committed osteoclast precursors by PTHrP (Hulter *et al.* 1985).

Prostaglandins of the E series are powerful stimulators of bone resorption (Klein & Raisz 1970), although their role in bone destruction associated with malignancy remains unclear (Mundy 1995). Some of the effects of cytokines on bone may be mediated in part through prostaglandins. Indomethacin, a prostaglandin synthesis inhibitor, has been shown to block part of the osteoclast-stimulatory effects of IL-1 *in vivo* (Sabatini *et al.* 1988, Boyce *et al.* 1989). Although prostaglandins have been demonstrated to be produced by cultured tumour cells *in vitro*, indomethacin treatment of malignancy-associated hypercalcaemia is only occasionally effective (Mundy *et al.* 1983). Thus, it is unlikely that prostaglandins have a major causal role in hypercalcaemia associated with malignancy.

Treatment of hypercalcaemia of malignancy

The ultimate treatment of malignancy-associated hypercalcaemia is eradication of the underlying cancer. However, cure is frequently not possible, and in patients with symptomatic or life-threatening hypercalcaemia therapy must be aimed specifically against the mediating mechanisms. Increased osteoclastic bone resorption is present in essentially every patient with hypercalcaemia of malignancy and is, therefore, a key target for treatment and prevention of hypercalcaemia. Two of these mechanisms in particular, increased osteoclastic bone absorption and increased renal tubular calcium reabsorption, are common to most patients with hypercalcaemia of malignancy, even those cases not associated with PTHrP production (Tuttle *et al.* 1991). Medical therapy is, therefore, aimed at inhibiting bone resorption and promoting renal calcium excretion. As many hypercalcaemic patients are dehydrated at presentation, the latter can be effectively

addressed with intravenous administration of isotonic saline. This first step of therapy serves to rehydrate the patient while enhancing calciuresis by increasing the glomerular filtration rate and reducing the fractional reabsorption of calcium and sodium. The use of loop diuretics, such as furosemide, to enhance calcium excretion is frequently overemphasised in clinical practice. These agents may exacerbate fluid loss; therefore, their use should be limited to the volume-repleted patient and only then with close monitoring of volume status (Suki *et al.* 1970). Hydration alone rarely results in full resolution of hypercalcaemia (Hosking *et al.* 1981), however, and more aggressive therapies are usually needed.

Bisphosphonates are currently the mainstay for long-term treatment of hypercalcaemia and osteolytic bone disease. They have an affinity for bone surfaces undergoing active resorption and are released in the bone microenvironment during remodelling. These compounds decrease osteoclastic bone resorption by two described mechanisms. First, the nitrogen-substituted bisphosphonates, such as alendronate, risedronate and zoledronic acid, are potent inhibitors of the enzyme farnesyl diphosphate synthase, thereby blocking protein isoprenylation. It is believed that the prenylation of small GTP-binding proteins is important for structural integrity of the osteoclast; without it, the osteoclasts undergo apoptosis. Secondly, the non-nitrogen-containing bisphosphonates, clodronate and etidronate, are less potent and also induce osteoclastic apoptosis but by a different mechanism. These bisphosphonates are metabolically incorporated into non-hydrolyzable ATP analogues that inhibit ATP-dependent intracellular enzymes. The bisphosphonates have been known to inhibit osteoclastic bone resorption for over 30 years.

There are also several lines of evidence that suggest bisphosphonates effect osteoclastic bone resorption indirectly through actions on osteoblasts. For example, bisphosphonates have previously been shown to have multiple actions on osteoblasts such as (1) modulation of proliferation and differentiation (Reinholz *et al.* 2000), (2) prevention of apoptosis (Plotkin *et al.* 1999), (3) modulation of extracellular matrix protein production (Giuliani *et al.* 1998b, Klein *et al.* 1998), (4) regulation of the expression and excretion of IL-6 (Giuliani *et al.* 1998a, Tokuda *et al.* 1998) and (5) decrease of angiogenesis (Green & Clezardin 2002, Wood *et al.* 2002). Recently, Viereck *et al.* (2002) presented *in vitro* evidence that bisphosphonates act directly on human osteoblasts to increase the production of both OPG mRNA and protein. Clearly, the implication is that by increasing OPG expression,

the RANKL-mediated stimulation of osteoclasts can be neutralised.

The various bisphosphonates available on the market vary in potency, but all are poorly absorbed from the gut and are most effective for treatment of hypercalcaemia when used intravenously. Intravenous etidronate, the least potent of its class, normalized calcium concentration in 30–40% of patients when given in doses of 7.5 mg/kg on 3 consecutive days (Kanis *et al.* 1987, Singer *et al.* 1991, Gucalp *et al.* 1992). Oral etidronate, at dosages of 25 mg/kg per day for more than 6 months can cause bone mineralization defects (Fleisch 1991). Pamidronate, alternatively, combines high potency with low toxicity and has become the agent of choice for the treatment of hypercalcaemia of malignancy. When used in the recommended doses of 30–90 mg i.v. over 4–24 h, it is highly effective in normalizing serum calcium concentrations and is not associated with mineralization defects. The onset of clinically apparent action is somewhat delayed with the bisphosphonates. Clinical studies using 90 mg infusion of pamidronate over 4 h indicate that the mean time to achieve normocalcaemia is approximately 4 days, while the mean duration of normocalcaemia is 28 days (Purohit *et al.* 1995). An effective method for achieving a rapid and sustained reduction in serum calcium concentration is to use pamidronate in combination with calcitonin. The combined use of a bisphosphonate with calcitonin lowers serum calcium levels more rapidly and effectively than either alone (Thiebaud *et al.* 1990).

Zoledronic acid, the most potent bisphosphonate available, has been recently approved for the treatment of hypercalcaemia of malignancy. At a dose of 4 mg and 8 mg, this bisphosphonate was compared with a single infusion of pamidronate (90 mg) in patients with hypercalcaemia of malignancy. Zoledronic acid was superior in respect to response rates, time to calcium normalization and response duration (Major *et al.* 2001). The 4 mg dose is Federal Drug Administration approved, as renal dysfunction occurred with the 8 mg dose.

Inhibiting osteoclastic bone resorption and renal tubular calcium reabsorption within minutes of administration, calcitonin also makes an excellent agent for the acute treatment of hypercalcaemia. Unfortunately, tachyphylaxis frequently develops within 48 h as a result of down-regulation of the calcitonin receptor. Concomitant use of glucocorticoids, however, can prolong the effective time of treatment (Binstock & Mundy 1980). This effect appears to result from a glucocorticoid-mediated up-regulation of cell-surface calcitonin receptors and increased *de novo* production

of calcitonin receptors in the osteoclast (Wada *et al.* 2001). Calcitonin can be administered every 6–12 h in doses of 4–8 U/kg. Human calcitonin is available but salmon calcitonin is generally used. If salmon calcitonin is used, then a test dose of 1 U should be administered first, since rare anaphylactic reactions have been reported.

Two anti-neoplastic agents have been used effectively for the treatment of malignancy-associated hypercalcaemia. Plicamycin, or mithramycin, is an inhibitor of DNA-dependent RNA synthesis and a potent inhibitor of bone resorption. The dosage used to treat hypercalcaemia (25 µg/kg) is one-tenth the usual chemotherapeutic dose and should be infused over 4 h. It has considerable side-effects of nephrotoxicity, hepatotoxicity, thrombocytopenia, nausea and vomiting. Gallium nitrate is another anti-neoplastic agent with calcium-lowering effects. It also has serious nephrotoxicity and is somewhat inconvenient to use as it is administered as a continuous 5-day infusion. Use of these anti-neoplastic agents should be reserved for cases of hypercalcaemia that are unresponsive to maximum doses of bisphosphonates.

Finally, about 30% of patients treated with glucocorticoids for malignancy-associated hypercalcaemia respond with a fall in serum calcium concentration. Glucocorticoids are most likely to have a clinical effect in the setting of hypercalcaemia associated with multiple myeloma or haematologic malignancies associated with 1,25-(OH)₂D₃. In these situations, glucocorticoids inhibit osteoclastic bone resorption by decreasing tumour production of locally active cytokines in addition to having direct tumorolytic effects (Mundy *et al.* 1978). Glucocorticoids, in dosage equivalents of 40–60 mg prednisone daily, should be given. If no appreciable response is observed within 10 days, then glucocorticoid therapy should be discontinued.

Metastatic cancer and localised bone destruction

The potential for tumour metastasis, especially to bone, is greater with certain types of cancers. Lung, breast and prostate cancers all frequently metastasise to bone, and bone metastases are present in nearly all patients with advanced breast or prostate cancer. Bone is the third most common site of metastasis of solid tumours after the liver and the lung. Metastatic bone disease is generally divided into osteoblastic and osteolytic disease, but most cancers lie within a spectrum of these two extremes. Osteolytic metastases

are much more common, however, and are one of the most feared complications of malignancy. They are usually destructive and are much more likely to be associated with pathological fracture and hypercalcaemia. The consequences for the patient include intractable bone pain at the site of the metastasis, pathologic fracture after trivial injury, nerve compression syndromes due to obstruction of foramina (the most serious example is spinal cord compression) and hypercalcaemia, when bone destruction is advanced. Once tumour cells are housed in the skeleton, curative therapy is no longer possible in most patients and only palliative therapy is available. Tumour cells metastasise most frequently to the axial skeleton, and particularly to the vertebrae, pelvis, proximal ends of the long bones and skull (Galasko 1981). It is clear that there are important properties of both the tumour cell (the seed) and the skeleton (the soil) that determine the likelihood that any particular tumour will metastasise to bone.

The mechanism by which a solitary tumour is able to escape and invade other distant structures is beginning to be understood. Once tumour cells enter the circulation, they traverse vascular organs, including the red bone marrow, where they migrate through wide-channeled sinusoids to the endosteal bone surface. Since hypercalcaemia of breast cancer is associated with extensive bone metastasis in the majority of patients, understanding the mechanism for tumour cell migration to bone and subsequent bone destruction should also clarify the mechanisms by which cancer cells cause hypercalcaemia.

Clinical features

Bone pain is a frequent cancer-related complication with the spine being a common location of metastasis. As this is often an initial symptom, distinguishing metastatic bone disease from common causes of back pain such as disc disease and muscle strain can be difficult. However, there are warning signs that should alert the physician that the patient's discomfort could be due to a serious condition. Progressive pain in an older individual or a patient with a history of cancer warrants further investigation. One study evaluated the aetiology of back pain in 1975 patients in a primary care setting and discovered that 0.66% of patients had a malignancy (Deyo & Diehl 1988). Other worrisome signs and symptoms include age greater than 50 years, weight loss, no relief with bed rest and duration greater than 1 month (Deyo & Diehl 1988). A patient who is found to have lower extremity motor nerve dysfunction, dysreflexia or loss of bowel or bladder function

requires prompt evaluation for cord compression or spinal root impingement (cauda equina syndrome).

Pathological fractures as a result of metastatic bone disease result in acute pain and disability. Vertebral crush fractures will often result in height loss and pain, and can be the initial symptom of bone metastases. Fracture of other weight-bearing bones such as long bones and the hip result in the most disability.

Diagnosis

The diagnosis of metastatic bone disease often relies on radiographic methods followed by biopsy of the area in question, especially in a patient without an established diagnosis of malignancy. For patients with high clinical suspicion, plain radiographs are indicated for initial evaluation of focal bone pain. Plain films have a reported sensitivity of 60% and specificity of 99.5% for diagnosing vertebral metastases (Deyo & Diehl 1988). Although most lesions are described as either lytic or sclerotic, in reality skeletal metastases are typically mixed, and plain radiographs of affected areas are often abnormal. Radionuclide bone scan can confirm the diagnosis as the nuclear tracer, typically technetium-99, has affinity at sites of active bone formation, whether the sites are lytic or sclerotic. Bone scans are more sensitive than plain films for osteoblastic metastases, because increases in blood flow and bone mineral turnover that are detected by bone scans are evident earlier than radiographic evidence of overt bone remodelling (Jarvik & Deyo 2002). With the diagnosis of a malignancy that has a predilection for bone, radionuclide bone scans are the diagnostic test of choice for determining the stage of the disease. Magnetic resonance imaging (MRI) can also be utilised when results of plain films or bone scans are uncertain. They are particularly helpful at imaging soft tissues surrounding suspected bone metastases. The sensitivity of MRI for the diagnosis of spine metastases is 83–93% with a specificity of 90–97% (Jarvik & Deyo 2002). Furthermore, the use of diffusion-weighted and contrast enhancement can accurately differentiate benign from malignant processes (Spuentrup *et al.* 2001, Chen *et al.* 2002b). In a patient with suspected cauda equina syndrome, MRI provides excellent views of the vertebral column and spinal cord.

While laboratory studies are unable to diagnose skeletal metastases definitively, several are helpful when monitoring progression in established cases. A marked elevation of alkaline phosphatase in a patient with malignancy would raise the possibility of hepatic or skeletal involvement. An increase in the

bone-specific alkaline phosphatase would indicate an increase in bone mineral turnover and the likely presence of skeletal metastases. Peptide by-products produced during the formation of collagen are helpful in monitoring osteoporosis therapies and may also be beneficial in monitoring the progression of skeletal metastases. The major organic component of bone matrix is type I collagen. The complex biosynthesis of collagen by the osteoblast releases soluble pro-peptide fragments, which can be assayed as markers of new bone formation and osteoblastic activity. Similarly, osteoclastic bone resorption releases fragments from cross-linked collagen, which can be assayed as markers of bone destruction. These markers can be used to monitor active bone remodelling in patients and their responses to bisphosphonate treatment (Garnero 2001). Bone formation markers include bone-specific alkaline phosphatase and serum procollagen I amino-terminal propeptide, while resorption markers include urinary collagen cross-linked N-telopeptide (NTX), collagen I carboxy-terminal telopeptide, pyridinolines and deoxypyridinolines. Bone formation and resorption markers are often increased in prostate cancer patients with osteoblastic metastases. However, such markers, especially those of resorption, can also be increased as a consequence of bone loss due to androgen deprivation therapy and cannot be used alone to diagnose bone metastases. For example, NTX is not as sensitive as a bone scan for the diagnosis of bone metastases, but may provide an auxiliary diagnostic index for a bone scan (Fukumitsu *et al.* 2002). There is evidence, however, that markers of bone turnover can aid the clinician in determining the response to therapy (Costa *et al.* 2002). NTX is the most sensitive in that respect (Berruti *et al.* 2002).

Basic research on mechanisms responsible for osteolytic and osteoblastic metastasis to bone

Introduction: Paget's 'seed and soil' hypothesis

Hypercalcaemia of malignancy clearly conveys a poor prognosis, with survival of less than 3 months (Ralston *et al.* 1990, Wimalawansa 1994). Most patients with metastatic bone disease fortunately do not have hypercalcaemia and may survive for several years after the first detection of metastatic disease. The morbidity associated with metastasis to bone, however, is high. Bone pain, fracture, nerve compression and hypercalcaemia are common sequelae. Nearly 70% of women who die of breast cancer have bone metastasis

(Coleman & Rubens 1987). A large number of patients struggle for years with the complications of bone metastasis and therefore understanding the process is important to improve therapy and prevention. The remainder of this article will focus on the recent developments in our understanding of the mechanisms of osteolytic and osteoblastic lesions.

The process of metastasis is an extremely complex cascade of linked sequential events, each of which must be successfully completed for a tumour cell to establish a secondary tumour in bone.

After growth of a tumour at the primary site, a tumour cell must (1) detach from the primary site, (2) enter the systemic vasculature via the permeable neo-vasculature of the tumour, (3) survive host immune response and physical forces in the circulation, (4) arrest in a distant capillary bed, (5) escape the capillary bed and (6) proliferate in the metastatic site. Both entry and egress from the vasculature involve similar processes of attachment to the basement membrane, secretion of proteolytic enzymes in order to disrupt the basement membrane and migration through the basement membrane. The attachment of tumour cells to basement membranes and to other cells is mediated through cell adhesion molecules. Inherent tumour cell motility in response to chemotactic stimuli is also an important factor for tumour cell invasion of the secondary site.

Breast cancer is one of a limited number of primary neoplasms that display osteotropism, an extraordinary affinity to grow in bone. This property has provided a key paradigm for our understanding of the metastatic process. Paget, during his observations of breast cancer in 1889, proposed the 'seed and soil' hypothesis to explain this phenomenon. 'When a plant goes to seed, its seeds are carried in all directions; but they can only grow if they fall on congenial soil.' In essence, the microenvironment of the organ to which the cancer cells metastasise may serve as a fertile soil on which the seeds (or cancer cells) may grow. This century-old concept remains a basic principle of our understanding of cancer metastasis, guiding current progress in the research of molecules produced by bones and tumour cells to enrich the vicious cycle (Chirgwin & Guise 2000) of secondary tumour growth.

A comprehensive investigation to identify gene products that enhance breast cancer metastatic potential demonstrated that patients who develop metastases possess unique gene expression profile signatures and are predictive of aggressive disease (van't Veer *et al.* 2002). The gene products found to be over-expressed belong to families that control cell cycling, angiogenesis and invasion. A similar study using gene

expression profiling was reported but focused on gene products that have a specific role in bone metastasis. The human breast cancer cell line MDA-MB-231 forms osteolytic bone disease when introduced into athymic mice by intracardiac inoculation (Guise 1997). Subpopulations of MDA-MB-231 having a greater osteolytic potential than the parental cell line were isolated by serial passage and gene expression profile comparison with the parental cell line was performed (Kang *et al.* 2003). Eleven genes were identified that have a greater than fourfold expression pattern in the highly bone metastatic line. Four of these gene products, IL-11, connective tissue growth factor (CTGF), the chemokine receptor CXCR4 and matrix metalloproteinase (MMP)-1 were further analysed. Overexpression of not a single gene but of a combination of two or more in parental MDA-MB-231 enhanced *in vivo* osteolytic capacity. Thus, these genes that have different functions, i.e. chemotaxis, invasion and osteolysis, co-operate to produce a full bone metastasis potential. None of these genes was represented in a report by van't Veer *et al.* (2002) involving a gene expression study of primary tumours. This implied that breast cancer cells are capable of developing osteotropic potential rather than possessing inherent ability.

Chemotaxis, invasion and adhesion

Before establishing a metastatic lesion in bone or tissue, tumour cells must first adhere to extracellular matrix components and other cells. The predilection of certain tumours to metastasise to specific characteristic tissues is likely to be determined by ligand–receptor interactions between specific tumour cell type and target site (Roy & Mareel 1992). Indeed, experimental evidence supports the assertion that tumour cell surface expression of adhesion molecules mediates targeting to bone with subsequent development of metastasis (Yoneda 2000). A number of molecules have been identified that promote tumour cell escape, including E-cadherin, osteonectin, osteopontin and urokinase. However, the chemokine system, integrins and the MMPs have convincingly been demonstrated to play a more direct role in bone metastasis.

CXCR4

A study to identify potential chemokine receptors in the migration of breast cancer cells to metastatic sites reported that the chemokine receptor CXCR4 is highly expressed in these cells. Furthermore, the ligand, stromal cell-derived factor-1 (SDF-1 or CXCL12), was found to be present in tissues that represent

common sites of metastasis, including bone marrow. Moreover, neutralising antibodies to this receptor impaired metastasis to regional lymph nodes and lung in a mouse model, but bone metastasis was not examined (Muller *et al.* 2001). The expression of CXCR4 by breast cancer cells is regulated by other factors. Vascular endothelial growth factor (VEGF) functions as an autocrine factor in breast carcinoma cells and blockade of this signalling pathway decreases the invasiveness of the cancer cells *in vitro* (Bachelder *et al.* 2002). The transcription factor nuclear factor- κ B also controls CXCR4 expression by binding its promoter and activating transcription (Helbig *et al.* 2003). The CXCR4/SDF-1 interaction also acts in the homing to bone in other cancer cell types. CXCR4-expressing prostate cancer cell lines exhibited adherence to bone marrow endothelial cells when treated with SDF-1 and migrated across an SDF-1 gradient. This suggests a potential role of this interaction in prostate cancer bone metastasis (Taichman *et al.* 2002). CXCR4 may also be involved in bone marrow metastasis in neuroblastoma (Geminder *et al.* 2001).

Integrins

Bone marrow stromal cells express the vascular cell adhesion molecule-1 (VCAM-1), which is a ligand for $\alpha_4\beta_1$ integrin (Michigami *et al.* 2000). Tumour cells expressing $\alpha_4\beta_1$ integrin should preferentially adhere to bone marrow stromal cells to establish bone metastases. CHO cells transfected with $\alpha_4\beta_1$ integrin resulted in bone and lung invasion when inoculated intravenously into nude mice compared with lung invasion alone in mice inoculated with non-transfected CHO cells (Matsuura *et al.* 1996). Moreover, neutralizing antibodies against $\alpha_4\beta_1$ integrin or VCAM-1 inhibited development of bone metastases. Similar expression of $\alpha_3\beta_1$, $\alpha_6\beta_1$ or $\alpha_v\beta_1$ integrins did not induce bone metastases.

The $\alpha_v\beta_3$ integrin receptor that binds the Arg-Gly-Asp (RGD) peptide sequence is found on a variety of extracellular matrix proteins, including osteopontin, vitronectin and bone sialoprotein, and appears to be important in homing and, possibly, invasion of tumour cells into the bone endosteum (Sung *et al.* 1998, Felding-Habermann *et al.* 2001). Proliferation and adhesion of breast cancer cells appears to be controlled, at least in part, by the integrin $\alpha_v\beta_5$ (Sung *et al.* 1998). Although, prostate carcinoma cells express the integrin $\alpha_v\beta_3$ and others, antagonists of $\alpha_v\beta_3$ do not interfere with adhesion and the role of these molecules in prostate carcinoma bone metastases remains unclear (Cooper *et al.* 2003).

MMPs

The MMPs are a family of at least 28 zinc-dependent proteinases that either are bound to the extracellular membrane or secreted within the local environment (Egeblad & Werb 2002). MMPs participate in the progression of cancer metastases not only by the degradation of matrix leading to invasion, but also by the alteration of signalling molecules affecting tumour growth and migration. This process is manifested through the cleavage of tethered signalling molecules such as IGF-binding protein (IGF-BP)-1, E-cadherin, fibroblast growth factor receptor 1 and pro-TGF β (Stamenkovic 2003). MMP function is modulated by at least four tissue inhibitors of metalloproteinases (TIMPs) and α 2-macroglobulin. The expression of MMPs has been found to be increased in most cancer types including breast and prostate (Upadhyay *et al.* 1999, Bachmeier *et al.* 2001). High levels of MMPs have been associated with poor prognosis (Nakopoulos *et al.* 2003, Ranuncolo *et al.* 2003).

MMPs appear to have a specific role in bone metastases. MMP-2 and MMP-9 cleave latent TGF β -binding protein-1, which may result in the release of TGF β stored within the extracellular matrix of bone, thereby promoting cancer cell growth (Dallas *et al.* 2002). Blockade of MMP-2 activity decreases the invasiveness of breast and prostate cancer cell lines *in vitro*, possibly by interfering with the binding of bone sialoprotein of cancer cells with extracellular matrix integrin $\alpha_v\beta_3$ present within bone (Karadag *et al.* 2003). MMP-9 increases integrin $\alpha_v\beta_3$ -mediated migration of the breast cancer cell line MDA-MB-435 (Rolli *et al.* 2003) and neutralising antibodies to MMP-9 decrease the invasive potential of a prostate cancer cell line (Festuccia *et al.* 1999). Recently, MMP-3 and MMP-7 were reported to cleave cell membrane-associated RANKL to a soluble form resulting in osteoclast activation in an *in vitro* model, suggesting MMPs have a role in osteolytic bone metastases (Lynch & Matrisian 2003).

Other factors affecting chemotaxis, invasion and adhesion

Tumour cells metastasising from a primary site must have the motility to move through the bone marrow sinusoids in order eventually to arrest in bone. Autocrine motility factor (AMF), thymosin β 15 and heat-shock protein 27 (hsp27) have each been implicated as potential factors controlling cell motility (Watanabe *et al.* 1991, 1996). AMF is the extracellular form of a glycolytic enzyme, phosphoglucose isomerase. It is functionally similar to platelet derived-endothelial

cell growth factor (PD-ECGF), in being non-classically secreted and having distinct intracellular and extracellular functions (Jeffery *et al.* 2000). AMF has species-specific effects on bone cells, dose-dependently stimulating RANKL mRNA and depressing that of OPG in bone marrow stromal cells (Li *et al.* 2000). *In vivo*, however, it is not an osteolytic factor but stimulates periosteal bone formation in nude mice. Thymosin β 15 increases cell motility, and when production was decreased by expression of antisense constructs, metastases were prevented in the Dunning rat prostate adenocarcinoma model (Bao *et al.* 1996). Similarly, overexpression of hsp27 in MDA-MD-231 cells decreased cell motility *in vitro* and bone metastasis in mice (Lemieux *et al.* 1999). Several tumour factors have been described that have autocrine effects on tumour cell motility, including autotaxin (Nam *et al.* 2001) and AMF (Silletti *et al.* 1996). The latter is a well-characterised marker of metastatic breast cancer and its mRNA is increased by heregulin (Talukder *et al.* 2000). Increased activity of the non-receptor kinase c-Src has been associated with a breast cancer aggressiveness in human studies (Verbeek *et al.* 1996). In animal models, MDA-MB-231 clones with increased expression of c-Src produce larger osteolytic lesions and higher amounts of PTHrP compared with control MDA-MB-231 cells (Myoui *et al.* 2003). The increased metastatic potential may in part be due to modulation of tumour cell adhesion and motility by c-Src through integrin adhesion and signalling (Playford & Schaller 2004).

Local tumour syndromes in bone

Exploration of the pathophysiology of bone metastasis has been hindered by the paucity of animal models of spontaneous bone metastasis. Various techniques of experimental bone metastasis in animals have been developed throughout the years and include injection of tumour cells directly into (1) the intramedullary cavity (Galasko & Bennett 1976), (2) abdominal aorta (Powles *et al.* 1973), (3) tail vein with inferior vena cava occlusion (Shevrin *et al.* 1988), (4) left upper thigh muscle (Kostenuik *et al.* 1992), (5) left thoracic artery with renal artery occlusion and (6) left cardiac ventricle (Arguello *et al.* 1988, Nakai *et al.* 1992).

Osteolytic metastases

Secondary tumour deposition in bone frequently causes osteolysis or bone destruction at the site of deposition. Breast cancer is the most common tumour type to do so, although prostate, lung, renal cell and thyroid tumours are all associated with osteolytic

lesions. The following discussion will focus on breast cancer as a model for cancer-mediated osteolysis.

Breast cancer as the 'seed'. The first of these characteristics is the ability to cause the destruction of the hard, mineralized matrix. Breast cancer cells *in vitro* secrete abundant acid and proteolytic enzymes capable of destroying bone (Eilon & Mundy 1978). *In vivo*, however, it appears that tumour cells are not active effectors of bone destruction, particularly during the establishment of metastasis. Histological analysis and scanning electron microscopy of osteolytic bone metastases indicate that osteoclasts adjacent to tumour cells actively resorb bone (Boyde *et al.* 1986, Taube *et al.* 1994). This would suggest that breast cancer cells have the ability to stimulate osteoclastic bone resorption. The presence of PTHrP in primary breast tumour has been discussed previously. However, PTHrP expression by breast cancer at metastatic sites differs significantly from expression at the primary site. Retrospective studies of breast cancer metastasis revealed that 90% of metastases to bone express PTHrP, as compared with only 17% at non-bone sites and 60% of primary tumours (Southby *et al.* 1990, Powell *et al.* 1991, Vargas *et al.* 1992), suggesting that expression of PTHrP is a factor that favours metastasis to bone. This strong evidence linking PTHrP to osteolytic metastasis has led to speculation that PTHrP may be the sole factor responsible for osteoclastic activation at sites of secondary breast cancer deposition.

PTHrP as a mediator of osteolysis. Developing the theory of PTHrP as a mediator of osteolysis, Guise *et al.* (1996) tested the effects of a neutralizing antibody to PTHrP-(1-34) in a mouse model of bone metastasis. Inoculation of the breast cancer cell line MDA-MD-231 into the left cardiac ventricle of mice reliably produced osteolytic metastasis. Mice that were pre-treated with monoclonal antibody to PTHrP-(1-34) developed significantly fewer and smaller osteolytic lesions than controls. Histomorphometric analysis of long bones of mice treated with the PTHrP antibody revealed significantly fewer osteoclasts at the tumour–bone interface and less tumour than controls. Conversely, when MDA-MD-231 cells were engineered to overproduce PTHrP, an increase in the number of osteolytic metastases was seen (Guise *et al.* 1994). Additionally, when mice with established osteolytic metastases due to MDA-MB-231 were treated with the PTHrP antibody, there was an appreciable decrease in the rate of progression of disease as compared with control (Yin *et al.* 1995, Guise & Mundy 1996). Similar

findings have been demonstrated in this model using a human lung squamous cell carcinoma (Iguchi *et al.* 1996). The breast cancer cell line, MCF-7, does not express PTHrP and is not associated with osteolytic lesions. However, when engineered to overexpress PTHrP, MCF-7 cells induced marked bone destruction and increased osteoclast formation as compared with controls (Thomas *et al.* 1999).

The increased local PTHrP concentration drives RANKL expression and inhibits OPG secretion from osteoblasts and stromal cells, and thereby activates osteoclastogenesis through the RANK located on osteoclast precursors (Thomas *et al.* 1999). While PTHrP expression by tumour cells within the bone microenvironment results in osteolysis, PTHrP is not the primary factor that leads to the development of bone metastases. Murine mammary cancer cells, a tumour type that ordinarily does not produce PTHrP, overexpress PTHrP and develop hypercalcaemia but not bone metastases (Wysolmerski *et al.* 2002). Taken together, the data strongly suggest that PTHrP is not important in the establishment of osteolytic bone metastases but is critical in the progression.

Other factors. Tumour cells also produce a number of other important factors that lead to osteolysis. When IL-6, IL-11 and VEGF are secreted by osteolytic breast cancer cell lines following TGF β stimulation, they potentiate the effects of PTHrP on osteoclastic bone resorption (de la Mata *et al.* 1995, Kakonen *et al.* 2002a). IL-8 production correlates with an increased metastatic potential in MDA-MB-231 cells but appears to be independent of PTHrP secretion (Bendre *et al.* 2002). Bone-derived factors besides TGF β also contribute to this vicious cycle.

Factors opposing osteolysis. Just as tumour cells produce osteolysis-enhancing factors, they also produce other factors rendering the cancer less effective as a seed for metastasis. The breast cancer cell line MDA-MB-231, overexpressing either E-cadherin or TIMP-2, was studied utilising the mouse model of osteolytic metastases. This experiment resulted in a decrease in the development of osteolytic metastases compared with control non-transfected cells (Mbalaviele *et al.* 1996, Yoneda *et al.* 1997). Several interleukins, IL-4, IL-12 and IL-18, and interferon- γ inhibit osteoclastic bone resorption (Gillespie & Horwood 1998, Martin *et al.* 1998, Reddy & Roodman 1998). IL-18, in particular, has been shown to decrease osteolytic bone metastasis by using MDA-MD-231 cells in the mouse model (Nakata *et al.* 1999). It remains to be tested

experimentally, however, whether tumour cells metastatic to bone secrete factors that oppose osteolysis.

Bone microenvironment as the 'soil'. Immobilised within the mineralised bone matrix is a rich trove of growth factors, but the effects of bone-derived factors on tumour cells remain understudied. These growth factors are released from the matrix by osteoclastic bone resorption during the normal course of the physiological bone remodelling required to maintain structural integrity of bone. In addition to the immobilised growth factors within the matrix, cytokines and growth factors are also abundantly produced within the multicellular bone marrow, particularly by stromal and immune cells. Thus, once tumour cells arrest in bone, the high concentrations of cytokines and growth factors in the microenvironment provide a fertile soil in which to grow. The environment is further enriched as the tumour cells stimulate osteoclastic bone resorption, leading to the release of more bone-derived growth factors that enhance survival and growth of the cancer, while simultaneously disrupting normal bone remodelling thus resulting in bone destruction. van der Pluijm *et al.* (2001) elegantly demonstrated that several mRNAs are increased in bone versus non-bone sites of human breast cancer metastases in nude mice. RNA abundances were determined by species-specific RT-PCR. PTHrP, VEGFs and M-CSF were increased specifically in bone, while several mouse markers of host angiogenesis were similarly increased. These experiments did not identify the factor(s) responsible for the bone-specific mRNA induction.

TGF β . TGF β mobilised from the bone matrix increases metastasis of breast cancer by stimulating tumour production of PTHrP (Yin *et al.* 1999). Data from a renal cell carcinoma (Zakalik *et al.* 1992), a squamous cell carcinoma (Kiriya *et al.* 1993, Merryman *et al.* 1994) and the breast cancer cell line MDA-MB-231 (Guise *et al.* 1994) all suggest that TGF enhances PTHrP production post-transcriptionally, by mRNA stabilization. Pfeilschifter & Mundy (1987) showed that osteoclastic bone resorption releases TGF β in active form. TGF β normally stimulates mesenchymal cell proliferation and extracellular matrix biosynthesis, while inhibiting growth of epithelial cells (Massague 1998).

The effects of TGF β are mediated through complex receptor interactions (Massague *et al.* 1997). TGF β binds a type II receptor which recruits and phosphorylates a type I receptor in turn. MDA-MB-231 breast cancer cells were transfected with a cDNA

encoding a TGF β type II receptor lacking a cytoplasmic domain (T β RII Δ cyt) (Wieser *et al.* 1993). T β RII Δ cyt binds TGF β , but is unable to phosphorylate the type I receptor, thus signal transduction is not initiated and this mutant receptor acts in a dominant-negative fashion to block the biological effects of TGF β (Wrana *et al.* 1994). MDA-MB-231 cells so transfected did not increase PTHrP expression in response to TGF β stimulation when compared with controls. Similarly, when MDA-MB-231 cells expressing T β RII Δ cyt were inoculated into the left cardiac ventricle of mice, they caused both significantly fewer and significantly smaller osteolytic lesions compared with control cells (Yin *et al.* 1999). But when Wieser *et al.* (1995) performed the converse of this experiment by expression of a constitutively active TGF β type I receptor (T β RI(T204D)), this resulted in increased PTHrP production, marked osteolytic bone metastasis, and decreased survival. Finally, overexpression of PTHrP in MDA-MB-231 cells bearing the inactive T β RII Δ cyt receptor increased PTHrP production and accelerated the development of osteolytic metastasis (Yin *et al.* 1999). These experiments establish that both TGF β receptor activation and PTHrP are crucial for the development and progression of osteolytic bone metastasis. The TGF β signal is transduced by the p38 mitogen-activated protein kinase (MAPK) and the Smad pathways in the MDA-MB-231 breast cancer cell line. The combination of Smad dominant-negative blockade and p38 MAPK inhibition results in complete inhibition of TGF β -stimulated PTHrP production (Kakonen *et al.* 2002b).

Other osteolysis-stimulating factors. Osteoclastic resorption of bone releases high concentrations of ionised calcium and phosphate from the dissolution of the bone mineral. The CaR is a G protein-coupled, seven-transmembrane domain receptor, which responds to small variations in the concentration of extracellular calcium (Yamaguchi *et al.* 2000). The CaR is expressed by breast cancer cells and regulates tumour secretion of PTHrP (Buchs *et al.* 2000, Sanders *et al.* 2000), an effect which is enhanced by TGF β . Thus, the high concentrations of ionized calcium in bone may contribute to the vicious cycle by increasing PTHrP production and osteolysis. Small molecule agonists and antagonists of the receptor have been developed and are in clinical trials (Nemeth 2002). Such agents might be effective against breast cancer bone metastases. The IGFs are also released into the local bone environment during osteolysis and likely also have a role in the proliferation of bone metastases (Sachdev & Yee 2001, Yoneda *et al.* 2001). Hauschka

Figure 2 Bone resorption in breast cancer osteolytic metastases. PTHrP secreted by tumour cells increases RANKL and decreases OPG activity, resulting in increased osteoclastogenesis. This escalation in bone resorption leads to higher levels of TGF β , which activates tumour cells to produce even more PTHrP, thus the 'vicious cycle' of osteolytic bone metastases begins. Other factors immobilised in bone that are released during osteoclast-mediated resorption include members of the IGF family. IL-1, -6, -8 and -17 and MMPs are secreted by tumour cells and appear to participate and stimulate osteoclastogenesis. OCL, osteoclast; MT-MMP, membrane type-matrix metalloproteinase.

et al. (1986) found that IGF-II, then IGF-I, were the most abundant factors in bone matrix, followed by TGF β , after which were lower concentrations of BMPs, fibroblast growth factors-1 and -2 and PDGF. However, only TGF β has been shown to play a direct role in stimulating tumour cells.

Interactions between tumour and bone — the vicious cycle. Preclinical animal models have established that bone metastasis involves a vicious cycle between tumour cells and the skeleton (Fig. 2). The cycle is fueled by four contributors: the tumour cells, bone-forming osteoblasts, bone-destroying osteoclasts and organic bone matrix. Osteoclast formation and activity are regulated by the osteoblast, adding further complexity. The mineralised matrix of bone is a vast storehouse of growth factors, such as insulin-like and transforming growth factors (Hauschka *et al.* 1986). These are synthesised by osteoblasts and released by osteoclasts. The factors reach high local concentrations in the bone microenvironment and can act on tumour

cells to encourage metastatic growth. It was realized 25 years ago that the products released from resorbing bone were attractants for tumour cells (Orr *et al.* 1979). In turn, breast cancer cells secrete many factors that act on bone cells. It is likely that at sites of osteoblastic metastases tumour cells continue to secrete osteolytic factors, such as PTHrP, which stimulate bone resorption. Therapies targeting the vicious cycle would be expected to decrease metastases by lowering the concentrations of growth factors in bone.

Osteoblastic metastasis

Osteoblastic metastases are commonly associated with prostate cancer and to a much lesser degree with breast cancer. Osteoblastic bone lesions have been described in other malignancies such as an osteosclerotic variant of myeloma (Anonymous 1972), colon cancer (Paling & Pope 1988), astrocytoma (Kingston *et al.* 1986), glioblastoma multiforme (Gamis *et al.* 1990), thymoma (McLennan 1991), carcinoid (Giordano *et al.* 1994), nasopharyngeal carcinoma (Liaw

et al. 1994), leptomeningeal gliomatosis (Pingi *et al.* 1995), Zollinger–Ellison syndrome (Pederson *et al.* 1976) and cervical carcinoma (George & Lai 1995). Just as with breast cancer-mediated osteolysis, the seed and soil hypothesis applies to prostate cancer in that the bone microenvironment readily supports the growth of prostate cancer cells (Koutsilieris 1995). The key difference, however, is that prostate tumour cells secrete factors that stimulate bone formation rather than destruction.

The seed and soil analogy of Paget can be applied to osteoblastic metastases, which share a similar pathophysiology with osteolytic metastases. The tumour cells, or seeds, secrete factors that stimulate osteoblast activity and bone formation. The bone microenvironment is enriched with osteoblast-derived growth factors, which enhance the local growth of tumour cells. Histomorphometric studies of prostate cancer samples indicate that osteoblastic metastases are due to tumour-produced factors that stimulate bone formation (Charhon *et al.* 1983, Koutsilieris 1995).

Data such as these suggest that prostate cancer cells produce factors that stimulate dysregulated bone formation. The following tumour products have been proposed to be important in the genesis of the osteoblastic response to tumour cells in bone.

Endothelin-1 (ET-1). ET-1 is a 21 amino acid peptide found to be a potent vasoconstrictor. It is expressed by many tissues and binds to two G protein-coupled receptors. It is also a potent osteoblast-stimulatory factor through its activation of the ET A receptor (ETAR) (Guise *et al.* 2003). Many receptor-selective ET receptor antagonists have been developed by the pharmaceutical industry for the treatment of cardiovascular conditions (Remuzzi *et al.* 2002). ET-1 plays an important role in cancer and on osteoblastic bone metastases in particular (Nelson *et al.* 2003a).

We established an animal model of osteoblastic metastases using three standard human breast cancer cell lines, ZR-75.1, T47D and MCF7. All three lines are oestrogen receptor positive and express ET-1 but not PTHrP. The ZR75.1 cell line was studied in the greatest detail. It stimulates new bone formation in organ culture and causes osteoblastic metastases *in vivo*. Bone formation and metastases were effectively blocked with a selective antagonist of the ETAR (Yin *et al.* 2003). This orally active antagonist, atrasentan, is in clinical trials in men with advanced metastatic prostate cancer (Rosenbaum & Carducci 2003). The vicious cycle model (Fig. 3) predicts that osteoblasts, osteoclasts and tumour cells co-operate to cause the pathology of bone metastases. The ET receptor

Figure 3 Bone formation in prostate cancer osteoblastic metastases. Secreted products of prostate cancer cells that stimulate osteoblast proliferation and new bone formation have been identified and these include ET-1, fibroblast growth factors (FGFs), BMPs, adrenomedullin and PDGF. The tumour production of proteases, most notably PSA, cleaves PTHrP, TGFβ and IGF from its binding protein, resulting in the generation of fragments with osteoblast stimulating activity.

antagonist blocks the activation of osteoblasts by tumour-produced ET-1. It also decreases osteoclastic bone resorption, as indicated by decreases in markers of resorption seen in patient trials (Nelson *et al.* 2003b). Conversely, bisphosphonates effectively reduce skeletal-related events (SREs) in prostate cancer (Saad *et al.* 2002). The observations support a role for the vicious cycle in cancer patients. These results suggest that bisphosphonates should be effective therapy against all types of bone metastases and that ET receptor antagonists may be useful in the treatment of osteoblastic metastases due to breast cancer.

Other factors. Other factors responsible for osteoblastic metastases remain to be critically tested. Such factors need to meet two initial criteria: (1) ability to stimulate osteoblastic new bone formation and (2) expression by cancer cells. The bone morphogenetic proteins are obvious candidates, but a causal role in bone metastases has not been demonstrated. CTGF, identified in the experiments of Kang *et al.* (2003), is another factor that stimulates osteoblasts (Safadi *et al.* 2003). Adrenomedullin is a 52 amino acid vasoactive peptide with potent bone-stimulatory actions (Cornish & Naot 2002), which is produced by many cancers (Zudaire *et al.* 2003). We have unpublished data from lung and prostate cancer cell lines which suggest that adrenomedullin increases bone metastases *in vivo*. It is also an autocrine growth factor for breast cancer cells (Miller *et al.* 1996) and is transcriptionally regulated in endometrial cells by tamoxifen (Oehler *et al.* 2000). Its role in breast cancer bone metastases has not yet been investigated.

Mixed osteolytic–osteoblastic metastases are characteristic of both breast and prostate cancers. The effects of combined expression of osteolytic and osteoblastic factors on bone have not been studied, so the net response of bone at the metastatic site is unpredictable. Osteolytic factors such as PTHrP and IL-11 act on osteoblasts to increase expression of RANKL. We tested the effects of introducing the osteoblastic factor ET-1 into the PTHrP-secreting MDA-MB-231 breast cancer cell line. Instead of converting the bone response from osteolytic to osteoblastic, the bone-destructive effects were enhanced by ET-1 (JJ Yin, JM Chirgwin & TA Guise, unpublished observations). Some of this effect may be caused by autocrine responses of the tumour cells to ET-1. Osteoblastic factors can stimulate osteoblast proliferation, increasing the population of early osteoblasts (Coffman 2003). The enlarged pool of early osteoblasts responds to osteolytic factors by increased expression of RANKL (Deyama *et al.* 2000, Geoffroy *et al.* 2002). Recently Yi *et al.* (2002) overexpressed PDGF B-chain in MDA-MB-231 cells and observed osteosclerotic rather than osteolytic metastases.

A puzzling question has been the role of PTHrP in osteoblastic metastases, especially those due to prostate cancer, which nearly always express PTHrP. A partial explanation was provided by the observation that prostate-specific antigen (PSA) is a serine proteinase which cleaves PTHrP after residue 23 (Cramer *et al.* 1996, Iwamura *et al.* 1996). The resulting fragment fails to activate the classical PTH/PTHrP receptor. It was later observed that the inactive fragment PTHrP(1-16) increased cAMP in cardiomyocytes by activating the ETAR. Binding was attributed to a four amino acid near-identity between the two peptides (Schluter *et al.* 2001). We have extended these observations to bone. PTHrP(1-23) is a potent stimulator of calvarial new bone formation at concentrations as low as 1 nM. PSA is commonly expressed in breast cancer (Black & Diamandis 2000). The results suggest that PSA proteolysis, rather than inactivating PTHrP, converts it from an osteolytic factor to a potent osteoblastic one. This process may occur in breast cancer bone metastases. Proteolytic cleavage of IGF from its binding protein (Fielder *et al.* 1994) and the processing of latent TGF β to the active form (Killian *et al.* 1993) by PSA may also contribute to osteoblast stimulation.

Tumour cells may also secrete factors that oppose the development and progression of bone metastases. IL-18, which decreases osteoclast formation, is one such factor made by cancer cells (Nakata *et al.* 1999, Iwasaki *et al.* 2002). This unexplored territory may

reveal exciting new approaches for future anti-metastatic therapies. Similarly, the role of the immune system in bone metastases is understudied (Roodman 2003), as is angiogenesis (van der Pluijm *et al.* 2000).

Treatment of metastatic bone disease

Hormone therapies

The treatment of metastatic bone disease often requires a multidisciplinary approach, involving a medical oncologist, radiation oncologist and a surgeon. Systemic chemotherapeutic agents are often used and can have beneficial effects in bone metastases. Endocrine treatments for prostate and breast cancer are considered first-line therapies for tumours that are hormone responsive. Androgen blockade in men with advanced prostate cancer can reduce tumour burden and bone pain. The use of luteinizing hormone-releasing hormone agonists and anti-androgens are effective and are often preferred over older treatments such as orchiectomy. However, most prostate cancers will overcome androgen blockade after several years and transform to androgen independence. Selective oestrogen receptor modulators (SERMs) such as tamoxifen, and possibly raloxifene and toremifene, have benefit in the primary and secondary prevention of breast cancer (Anonymous 1988, Fisher *et al.* 1998, Pyrhonen *et al.* 1999, Cauley *et al.* 2001). Two aromatase inhibitors, anastrozole and letrozole, are approved in the USA for treatment of postmenopausal patients with advanced disease (Buzdar *et al.* 2001). These medications will likely replace SERMs as first-line treatment for breast cancer. Comparative clinical trials with tamoxifen demonstrate the superiority of aromatase inhibitors in length of disease progression and survival (Bonnetterre *et al.* 2000, Mouridsen *et al.* 2003, Nabholz *et al.* 2003). However as compared with tamoxifen, aromatase inhibitors resulted in increased bone resorption (Heshmati *et al.* 2002) and fracture risk (Howell *et al.* 2005).

External beam radiation

Prophylactic external beam radiation to prevent fracture in weight-bearing bones in conjunction with surgical fixation can prevent significant morbidity. The use of external beam radiation in the treatment of focal bone pain is also quite effective (Tong *et al.* 1982). Patients with extensive bone metastases can undergo hemibody irradiation but at the expense of increased toxicity. Controversy exists on the optimum dose and schedule of therapy. Multiple studies have examined whether higher doses of radiation with shorter

fractions are more beneficial than lower doses and more fractions; no study has, however, been entirely convincing. Recent meta-analysis (Wu *et al.* 2003) demonstrated no significant differences for pain scores using single or multi-fraction therapy as palliative treatment. A survey of American radiation oncologists revealed different practice methods among physicians in the USA and in Canada. Most radiation oncologists in the USA employ 30 Gy in ten fractions while in Canada, 20 Gy in five fractions is often used (Chow *et al.* 2000).

Spinal cord or cauda equina compression by a spinal metastasis is a medical emergency requiring prompt evaluation and treatment in order to preserve and protect neurological function. In addition to focal back pain, radicular symptoms and signs include lower extremity muscle weakness, loss of bowel/bladder function and ataxia. Plain films have limited value in this situation and evaluation with MRI or myelography is required to identify the presence of a spinal lesion and cord compromise. High-dose corticosteroids in conjunction with external beam radiation or surgical decompression can result in significant return of neurological function if treatment is initiated within 24–36 h of symptoms (Maranzano & Latini 1995). Until recently, laminectomy was the preferred management in patients with cord compression but studies have shown no differences in outcome with radiation therapy (Young *et al.* 1980). Therefore, radiation therapy with corticosteroids is considered first-line therapy.

Radioisotopes

Radioisotopes with an affinity for bone have been studied in patients with metastatic breast and prostate cancer with painful bony metastases. Phosphorus-32 had been used in the past but has been surpassed by newer agents. Strontium-89 (^{89}Sr) is a high-energy β particle-emitting radioisotope that has been shown to be effective in decreasing pain. In one study, patients with prostate cancer and metastatic bone disease were randomized to receive either ^{89}Sr or external beam radiation (focal or hemibody depending on the extensiveness of the metastases) (Quilty *et al.* 1994). Both treatments were highly effective, although there was no significant difference in pain relief scores. However, patients randomized to the ^{89}Sr treatment had fewer subsequent painful bony sites and this result was likely due to the systemic nature of this therapy. The radioisotope samarium-135 (^{135}Sm) is also effective in alleviating pain in bone metastases (Serafini 2000) and has several advantages over ^{89}Sr . ^{135}Sm has

a shorter half-life (2 days) and therefore the delivery of larger doses over a shorter amount of time is possible. This radioisotope emits a lower energy particle than other radioisotopes, which translates into reduced bone marrow toxicity.

Surgery

Prophylactic surgical correction of bone metastases is indicated for impending fractures in weight-bearing bones. Osteolytic lesions that encompass a large area of bone have a high likelihood of fracture with breast and kidney metastases having the highest risk (Higenbotham & Marcove 1965). Fracture from osteoblastic metastases from malignancies such as prostate is unusual. Although pathological fracture at any location can result in significant pain, fracture of the femur, humerus, pelvis and vertebrae results in the most disability and fixation at these locations is indicated. Management of impending fracture includes plate osteosynthesis, nailing and insertion of prosthetic implants (Harrington 1997). Adjuvant radiotherapy after fixation can reduce further metastatic destruction.

Bisphosphonates

Bisphosphonates are unique in the treatment of metastatic bone disease as this is a bone-targeted therapy. The use of bisphosphonates in the treatment of osteolytic bone disease due to breast cancer also appears to have benefit. Two large trials have been published evaluating pamidronate in patients with stage IV breast cancer; the data were later combined in order to evaluate long-term benefit (Lipton *et al.* 2000). Patients were randomised to pamidronate (90 mg) or placebo every 3–4 weeks, and the primary outcome was skeletal events per year and time to first SRE. Despite a significant number of participants not completing the study, the number of events per year in the treatment group was 2.4 compared with 3.7 in the control group. Also, the median time to the first SRE was longer in the treatment group.

A phase II trial evaluating the effective dose of zoledronic acid compared with pamidronate in patients with osteolytic lesions due to multiple myeloma or metastatic breast cancer was published in 2001 (Berenson *et al.* 2001). Zoledronic acid (0.4, 2.0 or 4.0 mg) was compared with 90 mg pamidronate administered every 4 weeks up to 10 months. The primary end-point was the need for radiation therapy to bone during the treatment period, whereby secondary end-points included SRE, bone mineral density and bone pain. The 2.0 mg and 4.0 mg zoledronic acid-treated groups had an equivalent rate of radiation therapy

requirement as the pamidronate arm, but the 0.4 mg zoledronic acid-treated group underwent radiotherapy at a higher rate. A follow-up phase III trial with a similar patient population compared zoledronic acid (4 mg and 8 mg) with pamidronate (90 mg) infusion given every 3–4 weeks for 24 months (Rosen *et al.* 2003). SREs were the primary end-point over 25 months. Due to a decline in renal function in patients randomised to 8 mg zoledronic acid, this dose was reduced to 4 mg. Zoledronic acid reduced the risk of skeletal complications by 16% compared with pamidronate. The oral bisphosphonate clodronate may also have benefit in preventing SREs (Kanis *et al.* 1996, Powles *et al.* 2002), but has not been approved for use in the USA.

The use of bisphosphonates in the treatment of prostate cancer has been controversial as bone metastases are usually osteoblastic and bisphosphonates exert their effects via osteoclasts. However, recent studies have shown a benefit. Patients with hormone-refractory metastatic prostate cancer were assigned to receive zoledronic acid (4 mg and 8 mg) followed later with 4 mg or placebo every 3 weeks for 18 months. Subjects who received zoledronic acid had less SREs and pain compared with the control group, but there was no significant difference in disease progression or performance status (Saad *et al.* 2002). Androgen deprivation therapy for prostate cancer can adversely affect bone mineral density and the indication for the use of bisphosphonates in this situation is clearer. One study in men with early stage hormone-responsive disease treated with androgen ablation therapy showed that 4 mg zoledronic acid administered every 3 months for 1 year had an increase in lumbar spine bone mineral density compared with a decrease in the control group (Smith *et al.* 2003).

Zoledronic acid has been effective in the treatment of bone metastases from other solid tumours, including lung, kidney and colorectal. In a recent phase III trial, 733 patients with metastatic bone disease were randomised to zoledronic acid (4 mg) or placebo. Infusions were administered every 3 weeks for a treatment duration of 21 months. The treatment group had a statistically significant reduction in time to first SRE but there was no effect in mortality (Rosen *et al.* 2003). Of note, in most bisphosphonate trials patients received supplementation with calcium and vitamin D. This often-neglected detail is important, since the use of bisphosphonates in the presence of vitamin D deficiency may impair bone mineralization.

A major morbidity for patients with skeletal metastases is intractable bone pain, which is reduced by bisphosphonate treatment (Heidenreich *et al.* 2001).

The mechanisms of pain caused by bone metastases are specific and complex. Factors produced by tumour cells, as well as molecules released by bone remodelling, stimulate pain receptors in bone (Mantyh *et al.* 2002). Interruption of the vicious cycle with bisphosphonates can thus reduce the concentration of pain-stimulating molecules in the microenvironment surrounding metastatic tumour cells.

Substantial controversy surrounds clinical data on the effects of bisphosphonates on breast cancer tumour burden at extra-skeletal sites (Boissier *et al.* 2000, Diel *et al.* 2000). As discussed above, bisphosphonates have direct anti-tumour actions *in vitro*, but it remains unclear whether the compounds reach sufficiently high local concentrations in non-bone sites to produce such actions in patients. The bisphosphonates have the potential to enhance anti-tumour activities of known cytotoxic agents, but further and larger clinical trials are required to address the importance of the pre-clinical data. *In vitro*, bisphosphonates have direct effects on tumour cells to reduce tumour growth (Lee *et al.* 2002) and invasiveness (Derenne *et al.* 1999, Virtanen *et al.* 2002), as well as to induce apoptosis (van der Pluijm *et al.* 1996, Senaratne *et al.* 2000). Bisphosphonates may also impact other components of the metastatic cascade by reducing adhesion of tumour cells to bone (Boissier *et al.* 1997, Oefelein *et al.* 2002) and by inhibiting angiogenesis (Fournier *et al.* 2002). In the latter study, zoledronic acid reduced testosterone-induced revascularisation of the prostate in rats, the effect of which was due to transient accumulation of the bisphosphonate in the prostate (Fournier *et al.* 2002).

Questions still remain to be answered about the use of bisphosphonates for metastatic bone disease: which is the best bisphosphonate, what is the ideal dose, for how long can it be administered, how should it be administered, should it be given to patients early in the course of the disease and, most important, do these drugs have a beneficial effect on survival? Also, what are the effects of bisphosphonates on the tumour cells themselves? Finally, what is the role of bisphosphonate therapy in the treatment of osteoporosis due to cancer treatment, independent of the presence of bone metastases?

ETAR antagonists

Recent data show an important role for tumour-secreted ET-1 in osteoblastic metastases. ET-1 is a potent stimulator of osteoblast proliferation and new bone formation. Tumours secreting ET-1 cause osteoblastic metastasis in an animal model and the

metastases can be blocked with an ETAR antagonist (Yin *et al.* 2003). This antagonist, atrasentan, has completed a phase II clinical trial in men with hormone-refractory prostate cancer (Carducci *et al.* 2003). The agent delayed time to progression and decreased markers of tumour burden. Of particular interest, atrasentan decreased markers of bone remodelling, which were elevated in patients with prostate cancer (Nelson *et al.* 2003b). This result supports the importance of the vicious cycle model, in which targeting one step serves to suppress all parts of the cycle. Two other ETAR-selective antagonists, YM 598 and AZD 4054, are in clinical trial for treatment of prostate cancer (Nelson *et al.* 2003a). Other selective and non-selective ET receptor antagonists have been developed and are being investigated for treatment of pulmonary hypertension, congestive heart failure and chronic renal failure (Remuzzi *et al.* 2002).

Novel therapies

Although the current therapy for bone metastases results in a significant reduction in morbidity, such therapy does not promote regression of established disease. Thus, new therapies are under development. Novel therapies for osteolytic bone disease based on inhibition of the RANK/RANKL system have been proposed and several are in clinical trial. A phase I trial studying recombinant osteoprotegerin (AMGN-0007) in patients with multiple myeloma or bone metastases from breast cancer was recently published (Body *et al.* 2003b). However, concerns remain about adverse effects and theoretical concerns of decreasing cancer cell apoptosis. OPG is also a receptor for the TNF-like ligand TRAIL and this molecule has been shown to be involved in apoptotic signalling in cancer cells. A humanized RANKL antibody, AMG-162, has been developed and has shown promise in phase I clinical trials. The biological half-life after a single subcutaneous injection was at least 2 months and adverse effects were minimal. In breast cancer patients with metastases to bone, AMG-162 reduced urinary markers of bone resorption (Bekker *et al.* 2004). Integrins also appear to be important in the development of bone metastases (Bakewell *et al.* 2003). Mice injected with melanoma cells that result in osteolytic metastases had increased survival when treated with a monoclonal antibody to the integrin $\alpha_v\beta_3$ (Sparks *et al.* 2003). Integrin inhibitors are currently in phase II human trials (Tucker 2003). Vitamin D analogues have shown promise in preclinical models of breast cancer metastasis to bone, the mechanism of which is unclear but may involve inhibition of tumour growth or of PTHrP

production. Humanised PTHrP antibodies are in clinical trials for women with breast cancer metastatic to bone, but data regarding efficacy are not yet available. The tyrosine kinase c-Src promotes tumourigenesis and increases metastatic potential of tumour cells (Parsons & Parsons 2004). Mice inactivated for c-Src have osteopetrosis, demonstrating an additional role of this kinase in osteoclast function (Marzia *et al.* 2000). Inhibitors of this tyrosine kinase are being investigated for treatment of osteolytic bone metastases (Boyce *et al.* 2003).

Non-steroidal inflammatory agents (NSAIDs) and selective cyclo-oxygenase-2 (COX-2) inhibitors may reduce the risk of colon cancer (Thun *et al.* 1991, Rahme *et al.* 2003). This benefit may be related to increased expression of COX-2 in malignant versus normal colon epithelia (Eberhart *et al.* 1994). NSAIDs may also have a role in protecting against prostate cancer (Mahmud *et al.* 2004). Animal models of prostate cancer and sarcoma have shown a decrease in bone tumour burden with COX-2 inhibitors (Sabino *et al.* 2002, Gupta *et al.* 2004). The recent withdrawal of the COX-2 inhibitor rofecoxib, due to increased cardiovascular risk, may limit the future use of these agents.

Results based upon the exponential growth of the mechanisms underlying tumour metastases to bone indicate that many new therapies will be developed in the next 5 years. Possible targets include CTGF, CXCR4, MMPs, TGF β , IL-11, IL-8 and signal transduction inhibitors of the MAPK pathway. These new therapies may be useful in combination with the existing bisphosphonate treatments. Future preclinical and clinical trials will determine the importance of these newly identified targets in achieving regression of established bone metastases and in preventing the development of new disease.

Conclusion

Recent discoveries in the pathogenesis and treatment of bone metastases have been possible due to a more complete understanding of the molecular interactions between cancer cells and bone. Normal bone remodelling is complex and it has become clear that cancer cells with the propensity to flourish within the bone microenvironment have developed abilities not only to proliferate in bone but to coax osteoblasts and osteoclasts to produce factors within the bone microenvironment that further stimulate cancer cell growth. Thus, the development of treatments to break or at least reduce the 'vicious cycle' is key. Bisphosphonates have been shown to reduce SREs due to bone

metastases, but these drugs neither prevent metastases nor reduce cancer burden within bone. The majority of cancer therapies are aimed at systemic eradication of cancer cells but tailored therapies focusing on the treatment of specific cancers that metastasise to particular organs and tissues are the future of cancer therapy.

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