MECHANISMS OF LOCAL OSTEOLYSIS IN BONE METASTASIS: DIRECT ROLE OF CANCER CELLS AND THEIR MATRIX METALLOPROTEINASES

By

OTTO H. SANCHEZ-SWEATMAN, M.D., M.SC.

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OSTEOLYSIS BY CANCER CELLS AND THEIR MATRIX METALLOPROTEINASES

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AUTHOR:	Otto H. Sanchez-Sweatman, M.D., M.Sc.
SUPERVISORS:	Drs. F. William Orr and Gurmit Singh
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ABSTRACT

Bone is a common site of metastatic involvement. The mechanisms by which bone metastasis occurs are partially elucidated. Interactions between tumor cells and the bone microenvironment are being scrutinized, since their unravelling could lead to novel therapeutic approaches targeting bone metabolism additionally to tumor cells. This thesis summarizes experimental efforts addressing the role of metastatic cells in directly causing local osteolysis. Traditionally it has been thought that tumor cells, once in the bone, can only cause bone degradation by paracrine activation of osteoclasts. This work demonstrates that cancer cells are capable of degrading bone matrix and that their matrix metalloproteinases are partially responsible for this effect. The evidence reviewed here provides *in vivo* observations suggesting that this may occur during late "osteoclast-independent" phases of experimental bone metastasis by murine B16/F1 melanoma cells. *In vitro* documentation of bone and bone-related matrix degradation was obtained using these metastatic murine cells. A similar approach with human cell lines obtained from bone metastases demonstrated that these cell lines produce osteolysis *in vitro*. Since type I collagen is predominant in bone matrix, the potential involvement of tumor-derived matrix metalloproteinases was addressed. These studies showed that, in addition to murine B16/F1 melanoma cells, human prostate PC-3 adenocarcinoma, SK-N-SH neuroblastoma and Hs696 adenocarcinoma cells all produce these enzymes. Neutralizing their activity abrogated matrix degradation by

melanoma cells. Induction of enzyme activity correlated with increase in the degradative ability of these cells. Additionally, B16/F1 and PC-3 cells degraded type I collagen, further implicating collagenases as mediators of these effects. These findings are of potential clinical use since they put forward the possibility of using inhibitors of matrix metalloproteinases, already in clinical trials for other neoplastic conditions, in addition to the currently used antineoplastic and antiosteoclastic approaches, for the prevention and treatment of bone metastases.

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A. INTRODUCTION

Metastasis, the process by which cancer cells spread from the primary site of origin to other organs, is the principal cause of morbidity and mortality in cancer patients. Bones, after the lymph nodes, lungs and liver, are sites often affected by secondary tumors (Mundy et al., 1995; Easty et al., 1980; Berrettoni et al., 1986; Tubiana-Hulin, 1991). Common epithelial malignancies from the breasts, prostate, thyroid, kidneys and lungs, and other neoplasms such as multiple myeloma and neuroblastoma, preferentially metastasize to bone (Zetter, 1990; Galasko, 1981; Tubiana-Hulin, 1991). The mechanisms involved in this organ-specific process are not fully elucidated (Zetter, 1990). Clarification of the pathogenesis of bone metastasis is critical for the successful development of novel prophylactic, diagnostic and therapeutic approaches (Fidler, 1991a).

Research on metastasis is currently focused on the evaluation of interactions between tumor cells and their habitat at primary and secondary sites (Mundy et al., 1995; Miller et al., 1990; Fidler, 1990; Liotta et al., 1991; Fidler, 1991). There is also emphasis on the potential role these interactions may have on the expression of oncogenes and tumor suppressor genes that affect the metastatic behaviour of cancer cells (Nicolson, 1991a; Steeg et al., 1991a; Nicolson, 1991a; Evans, 1991a). In this context, it has been acknowledged that the "autonomy" of tumor cells, as described in traditional textbooks, is an inappropriate concept: although tumor cells

function more autonomously than normal cells, they depend on extracellular influences and respond to them (Fidler, 1991b; Kerbel, 1989b). Thus, metastasis research has not only focused conceptually and experimentally on the intrinsic properties of metastatic cells, but also on microenvironmental characteristics of primary and secondary host tissues, that will largely determine the phenotype of metastatic cells and the progression of the disease (Fidler, 1990b; Liotta et al., 1991b). Clinical applications of this research aim at early prediction of metastatic development in individual cancer patients, detection of micrometastases and eradication of established metastasis (Liotta et al., 1991c).

In this context, this thesis experimentally addresses specific concepts on interactions between tumor cells and the host in bone metastasis, focusing in particular on osteolysis directly caused by metastatic cancer cells.

1. Bone metastasis: The clinical problem

Compared with primary bone malignancies, metastatic lesions comprise the vast majority of bone tumors. Postmortem studies of cancer patients have reported an overall incidence of bone metastasis as high as 85% in breast and prostate cancer patients (Berrettoni et al., 1986; Rubens, 1991). Bone metastases display distinct anatomical and pathological features that hint at underlying pathogenetic

mechanisms. For instance, the observation that some bones are more frequently compromised by metastatic lesions than others (Tubiana-Hulin, 1991), has been explained by the unevenness of skeletal blood flow distribution and the high blood flow at places were red bone marrow is present (Rubens, 1991). It is accepted that the most common dissemination path used by cancers into bones is haematogenous. In general, skeletal involvement tends to be more central than peripheral, preferentially affecting axial bones (Berrettoni et al., 1986). This preferential distribution is related to tumor type: breast cancers most commonly affect dorsal vertebrae, while lumbar vertebrae and skull are often affected by prostate and thyroid cancer, respectively (Galasko, 1981b). Histopathologically, it has long been recognized that bone metastases are locally associated with reactive bone remodelling, including formation of woven bone. In this regard, Galasko studied bone metastases from patients with a variety of malignancies, and observed that in contrast to non-tumoral bones, metastatic bones displayed a significant increase in the proportion of woven bone (Galasko, 1982).

The clinical syndrome associated with bone metastasis includes: (a) pain in 75% of patients, (b) pathological fractures in 30%, (c) hypercalcemia in 15%, (d) immobility due to effects on neural or muscular structures in 5% of patients, and often caused by spinal cord compression, and (e) haematological alterations secondary to invasion and replacement of normal bone marrow (Elte et al., 1986; Rubens, 1991; Nielsen et al., 1991). Pain may be disabling and caused by a variety

of mechanisms including stimulation of endosteal nerve endings, periosteal stretching, invasion into surrounding innervated tissues or presence of pathological fractures (Nielsen et al., 1991). These fractures are more often associated with metastases from breast cancer, followed in descending order of frequency by secondary tumors from kidneys, lungs, thyroid, lymphomas and prostate. The bones most fractured are long bones such as femur and humerus (Rubens, 1991; Nielsen et al., 1991). Hypercalcemia is a consequence of either local bone destruction or humorally-mediated increased bone resorption by tumor-derived factors (Walls et al., 1995; Mundy, 1990). It is clinically characterized by increased urinary calcium excretion which may lead to renal function impairment, together with low serum phosphate levels and normal or low serum levels of parathyroid hormone and 1,25-dihydroxy-vitamin D (Elte et al., 1986). At least 86% of bone metastatic lesions in breast cancer patients will show radiologically evident osteolysis (Hortobagyi, 1991).

Early clinical detection of metastatic lesions in bone is improving in parallel with radiologic and radioisotopic imaging technology. Despite their lack of specificity, isotope bone scans have been the method of choice to detect and screen for early bone metastases (Elte et al., 1986). Currently, however, magnetic resonance imaging is the most sensitive imaging technique for their detection (Traill et al., 1995). Complementary diagnostic approaches include conventional bone radiographs and computed tomography, alkaline and acid phosphatases for

osteoblastic and osteoclastic activity, respectively, and bone biopsy (Nielsen et al., 1991). In up to 80% of patients with bone metastases secondary to breast cancer and 70% of those from prostate cancer, serum alkaline and acid phosphatases are elevated (Nielsen et al., 1991). Despite this diversity of diagnostic tests, bone metastasis are often detected late in the process of metastatic development, since most lesions are discovered only when a sufficient number of cancer cells has seeded the bone marrow, proliferated and in many cases destroyed bone. Bone micrometastases constitute the earliest detectable precursor of clinical lesions, since they are formed by individual or small groups of tumor cells present in the bone marrow space. Up to 31% of breast cancer patients already have detectable tumor cells in bone marrow aspirates at the time of surgical removal of the primary tumor (Menard et al., 1994). The presence of these cells, however, does not necessarily result in clinically evident lesions (Radinsky, 1991).

Current therapeutic approaches to bone metastatic lesions can, at best, only provide palliative aid (Nielsen et al., 1991; Hortobagyi, 1991). Treatments have two general aims, namely to decrease the number or eliminate viable tumor cells in the bones, and to control the life-threatening appearance of hypercalcemia (Elte et al., 1986). Clinically, the goals are to relieve pain, to prevent development of pathological fractures, to improve function and to prolong survival (Nielsen et al., 1991). Current treatment options include (Houston et al., 1995; Kanis, 1995): (a) surgery, mainly to prevent and repair pathological fractures or spinal cord

compression; (b) radiotherapy, often provided postoperatively to inhibit local tumor regrowth and stimulate bone repair, or preoperatively to decrease local pain; (c) chemotherapy, partially effective in skeletal metastases from breast and lung primaries, achieving complete remission in only 7% of breast cancer patients (Hortobagyi, 1991); (d) endocrine therapy, indicated in hormone-responsive tumors such as breast and prostate, including, for instance, antiestrogens such as tamoxifen, or castration; (e) use of radioisotopes to systemically target cancer cells or bone with ^{131}iodine, ^{90}yttrium, ^{32}phosphorus or ^{89}strontium, and (f) use of bisphosphonates, synthetic agents introduced in 1979, which adsorb onto hydroxyapatite crystals, inhibiting bone resorption (Chambers, 1980), and are currently used in the treatment of metastasis-associated osteolysis (Kanis et al., 1991; Fleisch, 1991). Additional support includes analgesia, psychological and social care, and physical therapy (Nielsen et al., 1991). The presence of bone metastasis in cancer patients constitutes a negative prognostic factor. Survival after clinical detection of bone lesions ranges from 9 months to 4 years (Nielsen et al., 1991).

Thus, clinical experience emphasizes the need to further understand the pathogenetic mechanisms in skeletal metastasis. This could lead to the identification of process-limiting steps that may be therapeutically targeted at early stages of tumor progression. From the tumor biology perspective, it is important to unravel the pathogenesis of bone metastasis as a peculiar example of organ-

specificity (Zetter, 1990). Despite multiple hypotheses put forward, involving site-specific tumor cell growth, adhesion or chemotaxis (Matsuura et al., 1996; Liotta et al., 1991), the question of why common cancers preferentially metastasize to the bone has not been answered. Understanding this key feature of cancer dissemination could encourage the development of novel preventive or therapeutic strategies. For now, it seems clear that organ-specificity is dependent on local interactions between tumor cells and host organ components (Price, 1990). It has been proposed that organ-specific homeostatic growth factors (Radinsky, 1991), tumor-derived growth factors (Daughaday et al., 1991), endothelial (Lafrenie et al., 1992), parenchymal or stromal adhesion molecules (Matsuura et al., 1996), and/or chemotactic factors may locally promote metastatic development (Zetter, 1990).

2. Mechanisms of bone metastasis

Two leading schools of thought explain why some human cancers display tropism to sites such as the skeleton: the mechanical and the "seed and soil" theories (Fidler, 1991a; Zetter, 1990a; Berrettoni et al., 1986a; Fidler et al., 1978a). Currently, it is generally agreed that both mechanisms are likely involved and ultimately determine metastatic behaviour.

The "mechanical" ("vascular") theory

This theory proposes that primary tumors from organs with venous drainage connected with the skeletal vasculature spread hematogenously to bones through these vessels. In 1940, Batson provided experimental anatomical evidence implicating the distribution of the bone venous vasculature as the main reason for preferential dissemination of cancers to different skeletal areas (Batson, 1940). This seminal study has constituted the basis of the "vascular theory" of bone metastatic distribution. According to this theory, the preferential metastatic distribution displayed towards bone by different malignancies may be mainly produced by the vascular links between the primary organs and the skeleton. Further experimental support for this concept has since been gathered. For instance, Coman and deLong experimentally confirmed Batson's findings on rat and rabbit vertebral metastases, the number of which was increased by external abdominal pressure (Coman et al., 1951). A similar increase in metastasis was observed with human prostate PC-3 adenocarcinoma cells injected intravenously with simultaneous caval occlusion (Shevrin et al., 1988). Additionally, Arguello *et al.* observed that after injecting tumor cells in the arterial circulation, the presence of bone metastases correlated with skeletal sites rich in haematopoietic bone marrow (Arguello et al., 1988). Consequently, it is well accepted that most secondary lesions in the bones are a product of blood-borne dissemination (Easty et al., 1980). In fact, Haq *et al.* demonstrated that bone-metastasizing rat prostate

adenocarcinoma cells preferentially adhere *in vitro* to bone marrow-derived endothelial cells (Haq et al., 1992), and Nakai *et al.* showed that a synthetic antagonist to laminin, a glycoprotein of the vascular basement membranes, inhibits the formation of metastasis by human melanoma cells (Nakai et al., 1992). These studies identify interactions between bone metastatic cells and vascular components that support the validity of the vascular theory of skeletal metastasis.

The "seed and soil" theory

In 1889, Stephen Paget noted that cancers do not spread and colonize organs at random, but rather, there is a tumor-specific preference to metastasize to certain organs (Paget, 1889). From the post-mortem study of 735 cases of breast cancer, he concluded that the skeleton was, among other organs, a frequent metastatic site. To understand this phenomenon, he proposed that target organs exert a "soil" effect on the cancer cells as "seeds". This notion has been coined as the "seed and soil theory" of metastasis. This theory presumes that circulating cancer cells, initially arresting in several organs following blood flow, develop metastatic lesions only in those with a favourable local microenvironment. Regarding bone metastasis, this idea is supported by the fact that bone vasculature *per se* does not completely explain the osseous preference by common cancers (Berrettoni et al., 1986). As happens with other target organs, such as brain, kidneys, heart and skeletal muscle, the vascular anatomy and proportional blood

flow to the bones do not correlate with skeletal metastatic distribution (Murphy et al., 1988; Zetter, 1990). Specifically, although the skeleton receives 5 to 10% of the total cardiac output, many tumors, including for instance those of gastrointestinal origin, only rarely metastasize to bone.

Consequent to Paget's proposal, it has been suggested that skeletal metastasis is facilitated by the presence of factors in bone that locally promote the malignant phenotype of cancer cells (Gleave et al., 1991; Chackal-Roy et al., 1989; Strobel et al., 1989). Bones are metabolically active structures, with two different tissue compartments: the mineralized cortex and bony trabeculae, and the bone marrow. The bone marrow, the site where haematopoiesis occurs, constitutes a biochemically rich microenvironment. The mineralized matrix exhibits constant remodelling which requires active regulation by osteoblasts and osteoclasts. This process is tightly controlled by soluble regulatory peptides. Research in bone endocrinology has contributed to the identification of several hormones and growth factors as physiological regulators of bone metabolism. Systemic regulators include parathyroid hormone, 1,25$(OH)_2$-vitamin D and calcitonin (Mundy, 1990a). Locally, growth factors may be synthesized by bone cells and tumor cells (Daughaday et al., 1991) and act in paracrine or autocrine fashions (Mundy, 1990b). Extensive work by Centrella *et al.* and others, has identified growth factors essential for the regulation of bone physiology, including basic and acidic fibroblast growth factors, platelet-derived growth factor, transforming growth factor β, and

insulin-like growth factors I and II (Centrella et al., 1985; Canalis et al., 1989; Centrella et al., 1991; Canalis et al., 1991; Canalis et al., 1991; McCarthy et al., 1994; Centrella et al., 1994). These factors exert many effects in osteoblasts and osteoclasts. The entire nature of these effects and possible interactions between these factors are not completely elucidated (Mohan et al., 1991).

3. Mechanisms of osteolysis in bone metastasis

In the context of skeletal metastasis, bone matrix degradation is of common occurrence and a frequent cause of clinical symptoms. Although it is generally accepted that bone metastatic lesions are commonly associated with local bone destruction and formation (Easty et al., 1980), imbalance of these two processes leads to a predominance of clinically evident osteolysis. Osteolysis associated with skeletal metastasis may be induced by various mechanisms. Possible mechanisms by which metastasis-associated local osteolysis may occur have been identified. Osteoclastic activation has been, by far, the best characterized of these mechanisms. Specifically, tumor-derived osteoclast activators have been identified, including parathyroid hormone-related protein, transforming growth factors α and β, prostaglandins, lymphotoxin, and interleukins 1 and 6 (Garrett, 1993; Easty et al., 1980; Mundy, 1991; Powell et al., 1991). These studies have led to the "traditional" concept implicating osteoclasts as the main mediators of bone matrix degradation

during bone metastasis. Since osteoclasts do not degrade non-mineralized matrix, osteoblasts must first degrade osteoid to expose the bone matrix to osteoclastic activity (Chambers et al., 1984). Tumor-associated macrophages and cancer cells have also been proposed as direct effectors of bone lysis (Mundy et al., 1977; Mundy et al., 1978; Quinn et al., 1994; Quinn et al., 1992; Athanasou et al., 1992). However, the mechanisms by which this occurs have been only partially elucidated, with no recent reports addressing the roles of cancer cells in direct osteolysis (Kanis, 1995; Eilon et al., 1983). Additional less specific mechanisms, such as ischemic necrosis of bone, due to vascular involvement by tumor, have been proposed as commonly occurring in metastasis secondary to lung cancer (Teitelbaum et al., 1994; Cramer et al., 1981).

Osteoclasts as mediators of osteolysis

Indirect evidence for a role of osteoclasts in the development of bone metastasis was first introduced in 1964 by Ingall who showed that parathyroidectomy in rats, performed prior to intramedullary injection of fibrosarcoma cells, extended survival in comparison to animals who were operated after tumor inoculation (Ingall, 1964). Local interactions between tumor and bone cells were initially demonstrated in the 1970s by Powles *et al.* (Powles et al., 1976; Powles et al., 1973). Using human breast tumors and rat Walker 256 cells, these authors showed that not only could tumor cells induce *in vitro* ^{45}Ca-release from rat

fetal calvariae, but also that this effect was inhibited by aspirin and indomethacin. Furthermore, in 38 patients with breast cancer, bone degradation by the tumor cells *in vitro* correlated with development of bone metastasis *in vivo* (Powles et al., 1976). The inhibitory effects of aspirin and indomethacin were experimentally observed *in vivo*: these drugs administered prior to or after intra-aortic inoculation of Walker 256 cells, selectively inhibited hypercalcemia and the development of bone metastasis in rats. Thus, these observations demonstrated for the first time that: (a) tumor cells locally induce bone degradation, (b) this process can be inhibited by pharmacological agents blocking prostaglandin synthesis, and (c) there is correlation between *in vitro* and *in vivo* bone metastatic behaviour. These findings were further extended by Galasko and Bennett (Galasko et al., 1976b) who demonstrated an indomethacin-induced decrease in osteolysis and osteoclast number in rabbits with intramedullary VX2 tumor and by Gavrilovic *et al.* who showed that VX2 cells cleave type I collagen through the production of interstitial collagenases (Gavrilovic et al., 1985).

Osteoclasts degrade bone by a combined action of membrane proton-channels and released proteinases (Vaes, 1988; Teti et al., 1991). Proton channels mediate secretion of hydrogen ions which acidify the local extracellular milieu thus inducing demineralization of the bone matrix (Baron, 1989). Experimentally, isolated osteoclasts have been shown to induce *in vitro* the release of ^{45}Ca from rat bone fragments and of ^{3}H-hydroxyproline from rat bone fragments and rat osteosarcoma-

derived matrices (Blair et al., 1986). This bone collagen degradation is mediated by a combined action of osteoclast-produced acid and neutral proteinases, including cysteine proteinases such as cathepsins, matrix metalloproteinases such as interstitial collagenases and MMP-9 (Okada et al., 1995), and other proteinases such as the plasminogen activator-plasmin system (Vaes, 1988). However, the functional relevance of each has been only partially tested experimentally, yielding conflicting results that emphasize the acidic (Blair et al., 1986) or neutral milieu required by active enzymes. Regardless, the combined action of the proton pump and the enzymatic machinery allows these specialized cells to efficiently first demineralize the bone matrix by decreasing extracellular pH, and then degrade the matrix components (Holtrop et al., 1977; Gluck, 1992; Everts et al., 1992; Vaes, 1988).

Osteoclast activating factors have been described as soluble mediators of *in vitro* osteoclast activity, and were originally found as products of stimulated human leucocytes including lymphocytes. For instance, *in vitro* studies with myeloma cell lines or primary cultures, showed that cytokines such as lymphotoxin, and interleukin 1 and 6 are produced by these cells and may stimulate osteoclastic activity (Mundy, 1991). Other factors, including interleukin-6, prostaglandin E_2 and transforming growth factor α have been implicated also in the activation and proliferation of osteoclastic activity by melanomas (Hiraga et al., 1995).

Osteoblasts as mediators of osteolysis

Osteoblasts, traditionally recognized as the main synthesizers of bone matrix, may assist osteoclasts in degrading bone by synthesizing matrix metalloproteinases and degrading osteoid, thus exposing the mineralized surfaces to osteoclasts (Boyce, 1991; Vaes, 1988; Thomson et al., 1989; Overall et al., 1989). Rat and mouse osteoblasts and osteoblast-like cells have been shown to produce collagenases, urokinase-type plasminogen activator and their inhibitors (Jilka, 1989; Meikle et al., 1995; Otsuka et al., 1984; Thomson et al., 1989; Sakamoto et al., 1989; Rifas et al., 1989). Release and activity of these factors can be modulated *in vitro* by transforming growth factor β (Overall et al., 1989a), other bone-derived growth factors (Meikle et al., 1991), and tumor cell-derived factors (Ohishi et al., 1995). Additionally, osteoblasts produce interleukin-6 which stimulates osteoclast activity (Pyke et al., 1993).

Monocytes and macrophages as mediators of osteolysis

Monocytes were initially shown by Mundy *et al.* to degrade devitalized ^{45}Ca- and ^{3}H-proline-labelled bones *in vitro* (Mundy et al., 1977). This effect was inhibited by cortisol, conceivably by stabilization of lysosomal membranes leading to enzyme release inhibition. Although this degradative effect could not be confirmed in morphological studies (Chambers et al., 1984), it has recently received additional

experimental support by work of Athanasou and Quinn, who used tumor-associated macrophages derived from human breast cancer implants. Specifically, these authors have demonstrated that multinucleated giant cells derived from a human breast carcinoma were able to resorb cortical bone *in vitro* (Athanasou et al., 1989), a phenomenon directly stimulated by purified parathyroid hormone. In view of the histogenetic concepts regarding the origin of osteoclasts, it is possible that these multinucleated cells are fused mononuclear cells rather than osteoclasts. Although these two cell types are structurally similar, osteoclasts are highly polarized cells and specialize in degrading bone (Baron, 1989).

Tumor cells as mediators of osteolysis

The role of metastatic cancer cells on the direct degradation of osteoid and mineralized bone is not a settled issue. Early experimental *in vivo* observations by Shivas *et al.* (Shivas et al., 1963) showed intramedullary tumor destroying trabecular bone in the absence of osteoblasts or osteoclasts, after intraosseous injection of Brown-Pearce carcinoma tumor fragments. In the late 1970s, Mundy *et al.* published evidence demonstrating that human breast cancer cells (Eilon et al., 1978) and malignant histiocytes (Koeffler et al., 1978) could, by themselves, induce release of radiolabelled calcium from devitalized rat fetal calvariae *in vitro* (Eilon et al., 1983; Mundy et al., 1978). More specifically, these studies showed that human MCF-7 adenocarcinoma and various MDA-MB adenocarcinoma and ductal breast

cancer cells and their conditioned medium induced ^{45}Ca and ^{3}H-proline release from devitalized bone (Eilon et al., 1978). These results were reproduced using conditioned medium from cells isolated from a patient with histiocytosis X (Koeffler et al., 1978). Thus, these experiments showed that in the absence of viable bone cells, cancer cells could degrade mineralized bone. Furthermore, it was determined that increases of intracellular cyclic AMP levels in MCF-7 cells, induced by cholera toxin, prostaglandin E_1, isobutylmethylxanthine or 8-bromo-cyclic AMP, correlated *in vitro* with increased collagenolytic and bone-resorbing activity (Eilon et al., 1983).

Occasional clinicopathologic and experimental studies of bone metastasis have been reported in which either osteoclasts, osteoblasts or tumor cells are observed in the "front line" of bone degradation (Yoneda et al., 1994; Easty et al., 1980). For instance, Cramer *et al.* studied bone metastatic lesions in lung cancer patients, and observed that bone cell activity rather than the presence of cancer cells was more commonly associated with bone resorption areas (91% *vs.* 35% of 80 cases, respectively) (Cramer et al., 1981). These observations, reinforced by experimental observations in bone metastasis by VX2 squamous carcinoma cells in rabbits, lead Galasko to propose a "two-phase hypothesis" of tumor-associated osteolysis: an "osteoclast-dependent" phase is followed by an "osteoclast-independent" phase, in which tumor cells and/or tumor-associated macrophages can directly participate in the destructive process (Galasko, 1982; Berrettoni et al., 1986; Galasko, 1981; Galasko, 1981; Galasko et al., 1976; Galasko, 1976). This

"dual nature" of osteolysis is accepted but considered applicable only on a tumor-specific basis; namely, variations among different tumor types and subtypes occur (Easty et al., 1980). A universal role for osteoclast activation in metastatic lesions from common tumor types such as prostate, breast and lung cancers, remains to be demonstrated. In fact, based on their experience with metastatic lung cancers, Cramer *et al.* have put forward a plausible proposal suggesting that mechanisms of osteolysis depend on histological subtypes (Cramer et al., 1981).

Despite these advances, controversy still exists in regard to the relative prevalence of cellular mechanisms involved in metastasis-associated local osteolysis (Quinn et al., 1994; Yoneda et al., 1994; Easty et al., 1980; Nielsen et al., 1991; Mundy, 1991; Boyce, 1991; Rosol et al., 1992; Body, 1992; Bataille et al., 1992; Garrett, 1993; Francini et al., 1993). Questions regarding coexistence of these multiple cellular mechanisms, and their time sequence during the development of individual bone lesions, have not been experimentally answered. Although the discovery of tumor cell products which activate osteoclasts has defined and reinforced the role of these cells as cellular mediators, molecular mechanisms by which tumor-associated macrophages and tumor cells directly degrade bone are yet to be defined. Although it is speculated that cancer cells could acidify their extracellular environment and thus induce demineralization, it is not clear how this may be accomplished in the absence of proton pumps acting on

a "sealed area". Furthermore, it is not known how cancer cells would remove the released calcium from the extracellular space before toxicity ensues.

4. Proteinases and metastasis

Metastasis involves multiple cellular interactions between cancer cells, host stromal cells, and extracellular matrix components, including basement membranes (Fidler et al., 1978; Miller et al., 1990). Various mechanisms underlying these interactions have been described and experimentally demonstrated. Among them, one critical step required by cancer cells in this multistep process is invasion of primary and secondary organs (Kohn, 1991). Tumor cell invasion requires attachment and degradation of the surrounding extracellular matrix, followed by cell migration (Aznavoorian et al., 1993; Liotta et al., 1991).

Matrix degradation by cancer cells is catalyzed in a cell-type specific manner by a battery of enzymes that degrade various extracellular matrix components. The enzymes implicated in tumor invasion and metastasis are either glycosidases or proteinases (Mignatti et al., 1993; Pauli et al., 1988). These are endopeptidases biochemically classified according to the salient features at their active sites, be it the predominant type of amino acid residues or the metal requirement, as: (a) serine-proteinases, including plasminogen activator, plasmin, elastase and

cathepsin G, (b) cysteine-proteinases such as cathepsins B, H, L and D, and (c) metalloproteinases such as collagenases and stromelysins.

These enzymes may be derived from the tumor cells *per se* or from stromal cells from the host organ (van den Hooff, 1991). In turn, tumor-derived proteinases can be secreted or membrane-associated. In this context, Zucker *et al.* described the presence of metalloproteinase and cysteine proteinase activities in plasma membrane extracts from murine B16 melanoma cells (Zucker et al., 1985). As well, Whitelock *et al.* (Whitelock et al., 1991) described the presence of interstitial collagenases in plasma membrane extracts from rat mammary carcinoma cells.

Biology of matrix metalloproteinases

Classification and common characteristics. Matrix metalloproteinases are a family of at least fourteen enzymes capable of degrading various extracellular matrix components (Stetler-Stevenson et al., 1996; Birkedal-Hansen, 1995). They are classified into interstitial collagenases, gelatinases and stromelysins (Matrisian, 1990; Woessner, Jr. 1991). These enzymes differ in their substrates (Nagase et al., 1991), but share the following common denominators (Krane, 1994; Stricklin et al., 1988; Pauli et al., 1988):

(a) they are neutral enzymes, with optimal pH between 7 and 8;

(b) they contain and require tightly bound zinc at the active site;

(c) they require the presence of a divalent cation, such as calcium, in order to catalyze degradation; and

(d) they are secreted as latent proenzymes and activated extracellularly (Liotta et al., 1991a), or may be associated to the cell surface (Stetler-Stevenson et al., 1996).

Domain structure. Structurally, these enzymes show amino acid sequence homology among them and share various domains (Liotta et al., 1991a; Liotta et al., 1991a): (a) a zinc-binding domain, at the enzymatic active site (VAA.HELGH, positions 206-214 in MMP-1 (Docherty et al., 1990)), with high homology among all the members of the family. The paired histidine residues are essential to bind the zinc atom; (b) a "hemopexin-like" domain, with about 200 amino acid residues, which may play a role in binding to matrix components and thus determine the specificity of each enzyme, and (c) a "proenzyme" domain of about 90 amino acids, at the amino terminal end, which contains a "cysteine switch mechanism", by which an unpaired cysteine residue (PRCGVPD, position 82, in MMP-1 (Docherty et al., 1990)) binds to the zinc at the active site in the latent or zymogen state of the enzyme (Woessner, Jr. 1991; Liotta et al., 1991).

Regulation of matrix metalloproteinase activity. Expression and activity of matrix metalloproteinases are regulated at various levels including gene transcription, mRNA stability and translation, packaging, secretion, activation, inhibition and clearance of the enzymes (Frisch et al., 1989; Matrisian, 1990; Matrisian, 1994; Murphy et al., 1994). Regulatory mechanisms, however, differ among specific cell types and for each family member. Most work on modulation of collagenolysis has been performed in human fibroblasts (Overall et al., 1989a; Overall et al., 1989a; Circolo et al., 1991a; Frisch et al., 1989a; Edwards et al., 1987a) and a few cancer cell lines (Brown et al., 1990). Regardless, three major sites of regulation have been better characterized and studied. Transcriptional regulation has been well documented by Brinckerhoff *et al.* (Brinckerhoff et al., 1979) by describing phorbol ester modulation of collagenase release from human fibroblasts. Later, Brown *et al.* described a similar phenomenon in human cancer cell lines including HT-1080 fibrosarcoma, and A2058 and HT-144 melanoma cells (Brown et al., 1990). Extracellular proenzyme activation occurs by proteolytic loss of the "proenzyme domain", which can be induced *in vitro* by organomercurial agents or by limited proteolysis. These treatments dissociate the zinc/cysteine association, thus releasing the amino-terminal segment from the active site. The subsequent conformational rearrangement and activation will trigger an auto-proteolytic process by which the proenzyme domain is removed. Indirectly, regulation of matrix metalloproteinase activity can be exerted by growth factor-

mediated modulation of the synthesis and/or activity of the plasminogen-plasmin system (Ponta et al., 1994; Keski-Oja et al., 1991).

Once matrix metalloproteinases are activated, they may be neutralized by specific natural inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). These include glycoproteins that specifically bind and inactivate matrix metalloproteinases (Docherty et al., 1990). There are three different proteins grouped in this category (Woessner, Jr. 1991): TIMP-1, M_r 28,000, which preferentially binds to gelatinase B; TIMP-2, M_r 21,000, a non-glycosylated protein preferentially binding to gelatinase A, and with a 38% amino acid sequence homology with TIMP-1 (Woessner, Jr. 1991); and TIMP-3 or chIMP-3, a matrix-associated protein (Apte et al., 1995; Apte et al., 1996). TIMPs noncovalently bind to matrix metalloproteinases with high affinity and a 1:1 interaction. Further, there is a slight selectivity as to the type of enzyme each TIMP type will bind: TIMP-1 preferentially binds to gelatinase B, while TIMP-2 does so with gelatinase A (Murphy et al., 1994).

Cytokines and growth factors such as interleukin 1, tumor necrosis factor α, basic fibroblast growth factor and platelet-derived growth factor have been shown to upregulate MMP-1 gene expression in fibroblast, fibrosarcoma and osteoblast-like osteosarcoma cell lines (Unemori et al., 1991; Herlyn et al., 1991; Stricklin et al., 1988; Frisch et al., 1989; Woessner, Jr. 1991). In contrast, transforming growth

factor β has been consistently shown in human fibroblasts and cancer cell lines, such as human A2058 melanoma cells (Brown et al., 1990), to transcriptionally suppress growth factor-induced MMP-1 synthesis, to enhance TIMP induction by other agents, and to induce release of plasminogen activator inhibitor type I (Roberts et al., 1988; Edwards et al., 1987; Overall et al., 1989; Overall et al., 1989).

In vitro studies on the regulation of synthesis and release of matrix metalloproteinases by cytokines and growth factors has shown that these events are dependent on the cell line studied, the agent used and the exposure time (Brown et al., 1990). Although the basis for this "differential regulation" is unclear, it is presumed to depend on the intracellular signal transduction pathways triggered by each agent. For instance, cell signalling events responsible for inhibition of matrix metalloproteinase synthesis by transforming growth factor β1 require upregulation of the synthesis of *Fos* (Kerr et al., 1990), a transcription factor and proto-oncogene product (Travali et al., 1990; Hunter, 1991). Promoter regions required for enzyme regulation include (Woessner, Jr. 1991): (a) the phorbol ester responsive or TPA-responsive element (TRE), which consists of a binding site for the AP-1 protein complex, a heterodimer of *Fos* and *Jun*, with a leucine zipper mechanism (Hunter, 1991); (b) the TPA and oncogene responsive unit (TORU), located upstream from the TRE, a binding site for the transcription factor PEA3, and

(c) the "transforming growth factor β1 inhibitory element" (TIE), which binds to *Fos* (Kerr et al., 1990).

Additionally, insoluble parenchymal or stromal extracellular matrix components may, through adhesive interactions with tumor cells, modulate production of matrix metalloproteinases and TIMPs (Woessner, Jr. 1991). Emonard *et al.* described increased synthesis and activity of fibroblast MMP-1 after exposing fibroblasts *in vitro* to type I collagen gel or matrigel (Emonard et al., 1990). Similarly, Werb *et al.* described stimulation of synthesis and secretion of matrix metalloproteinases in fibroblasts by integrin receptor ligands such as anti-fibronectin receptor antibodies and GRGDSP (Gly-Arg-Gly-Asp-Ser-P) hexapeptides, collagen gels, SPARC (secreted protein, acidic and rich in cysteine, or osteonectin) and tenascin (Werb et al., 1990). It is presumed that these phenomena occur through ligand-mediated integrin activation (Akiyama et al., 1990; Albelda et al., 1990; Albelda, 1993; Ruoslahti, 1991), triggering of signal transduction pathways and ensuing transcriptional regulation of matrix metalloproteinase expression.

Role of matrix metalloproteinases in metastasis

Extensive research on matrix metalloproteinases and their inhibitors has revealed a multiplicity of roles in physiologic and pathologic processes. Regarding

tumor cell invasion and metastasis, experimental observations have determined that tumor- or host-derived enzymes are important during these processes. Accordingly, their pathogenetic relevance has been placed in the context of a sequence of events proposed by Liotta *et al.* as the "three-step theory" of tumor invasion. These steps include, in chronological order, adhesion of invading cells to extracellular matrix components, enzymatic degradation of this matrix and migration of the cells through the degraded stroma (Aznavoorian et al., 1993; Liotta et al., 1991).

Tumor cells require collagenases to invade surrounding extracellular matrices. Substantial emphasis has been placed on the role of gelatinases A and B in tumor cell invasion. Specifically, multiple studies have shown correlations between gelatinase secretion by cancer cells and invasion (Liotta et al., 1991a), as well as elimination of invasive properties by inhibitors of matrix metalloproteinases (DeClerck et al., 1991). Also, studies by DeClerck *et al.* (DeClerck et al., 1991) have shown that recombinant TIMP-2 inhibits *in vitro* type I and IV collagenolytic activity, extracellular matrix degradation and invasion by c-Ha-*ras*-1-transfected rat embryo cells and human HT-1080 fibrosarcoma cells.

In vitro and *in vivo* studies addressing the role of matrix metalloproteinases in metastasis have used genetic manipulation of tumor cell lines that aim at artificially modulating enzymatic or inhibitor gene expression, and is followed by evaluation of changes in metastatic behaviour. This work has supported a role for

matrix metalloproteinases and their inhibitors in metastasis (Liotta et al., 1991b). Khokha *et al.* reported an increase in the metastatic ability of murine Swiss 3T3 cells by downregulating TIMP translation (Khokha et al., 1989). Additionally, murine B16/F10 melanoma cells overexpressing TIMP-1 showed decreased ability to produce experimental lung metastasis (Khokha, 1994). Other workers have shown that transfection of the *ras* oncogene into rat embryo fibroblasts concurrently increases their type IV collagenolytic activity and metastatic capacity, and that both phenomena are inhibited by cotransfection of the adenoviral protein E1A (Liotta et al., 1991c).

5. Rationale and hypotheses

In the context of the "seed and soil" theory of metastatic spread, experimental observations in our laboratory have revealed that close functional interactions occur between cancer cells and the bone microenvironment (Orr et al., 1993; Orr et al., 1995). Specifically, it has been postulated that the bone microenvironment promotes in cancer cells an aggressive local behaviour which facilitates the development of metastatic lesions. An extensive number of experiments with mammary rat Walker 256 cells, known for their osteotropism *in vivo* (Kostenuik et al., 1992a), has revealed that these cells preferentially attach to resorbing bone (Magro et al., 1985) and that soluble resorption products are

chemotactic to these cells (Orr et al., 1990; Magro et al., 1985). Furthermore, rat Walker 256 and human breast MB-MDA-231 carcinoma cells, among others, displayed increased growth and proliferation in the presence of soluble factors resulting from bone resorption (Kostenuik et al., 1992b; Manishen et al., 1986b). Transforming growth factor β1 and type I collagen peptides have been identified as partial mediators of these effects (Orr et al., 1990; Millar-Book et al., 1990; Kostenuik et al., 1993).

The work described in this thesis focused on the interactions between cancer cells and the bone matrix, after blood-borne seeding has occurred. Specifically, it addressed the notion that cancer cells may be responsible for *direct* osteolysis, on the basis that they have the elements required for matrix degradation. Since 90% of the organic matrix in bone is constituted of type I collagen (Pauli et al., 1988), I examined the possibility of tumor-derived matrix metalloproteinases being one of the molecular mechanisms metastatic cells use to cause osteolysis locally. Furthermore, it dealt with the possibility of modulation (i.e. upregulation) of tumor-mediated degradation by bone-derived factors. Consequently, the following were my hypotheses:

A. Cancer cells degrade bone matrix.

B. Bone matrix degradation is caused by tumor-derived matrix metalloproteinases.

C. Tumor cell behaviour is regulated by bone microenvironmental factors.

These concepts *per se* are not novel. More than a decade ago, Eilon, Mundy and co-workers reported degradation of mineralized bone by human breast cancer cells *in vitro* and proposed that this effect was mediated by tumor-derived enzymes. However, tumor-mediated osteolysis has not been reevaluated following the surge of knowledge on tumor invasion and matrix degradation, the discovery of the wide diversity of mammalian proteinases and recent advances in the molecular biology of matrix metalloproteinases.

The manuscripts presented in this thesis represent, in chronological order, the experimental work related to the histomorphometric characterization of a murine model of bone metastasis, followed by the *in vitro* study of osteolysis by murine and human bone-metastasizing cells.

B. CHARACTERIZATION OF A MURINE ANIMAL MODEL OF BONE METASTASIS

1. Introduction

To evaluate pathogenetic phenomena during bone metastasis, experimental *in vivo* models of skeletal metastasis have been developed. In this project, the first step was to develop such a model that would consistently and reproducibly result, within reasonable time, in the development of bone metastasis. Additional requirements for this study included the potential use of different tumor cell lines, avoidance of early death due to excessive tumor burden in organs other than bones, and optimally, development of multiostotic tumor lesions.

In this regard, the study of bone metastasis has been, to some extent, limited by the availability of adequate *in vivo* models. To study pathogenetic phenomena, an "ideal" animal model of skeletal metastasis should allow, in a simplified manner, consideration of specific questions on tumor cell interactions with the bone microenvironment. Consequently, this model should fulfill the following requirements: (a) be reproducible, (b) permit the use of tumor cells of diverse origins, (c) be spontaneous, thus, allowing the study of metastatic *versus* primary tumors, (d) not induce any additional disruption of anatomical or physiological host features, and (e) be assessed with reproducible and standardized quantitative

endpoints. Unfortunately, few experimental models display these attributes. Currently, many models used are reproducible when performed by trained individuals. However, most of them do not allow the use of more than a few cell types, most induce experimental (*versus* spontaneous) metastasis, and most produce to some degree local anatomical or physiological alterations. Additionally, most cells used are from rodent rather than human origin. There are also concerns regarding the use of cell lines, rather than "primary" cultures, which distance the former from their original "metastatic phenotype". This has limited application of important conclusions to only specific cell lines. Thus, most models have limitations that to some degree limit extrapolation of findings to clinical situations. The analysis of human specimens of bone metastasis, although valuable, often allows the observation of only a particular stage of the disease process, thus limiting their use for identification of time-dependent interactions of pathogenetic importance.

Historically, various animal models of bone metastasis have been described. Initially, localization of cancer cells in the bone was achieved by intramedullary injection of cancer cells into the long bones or ilium of rabbits and rats (Shivas et al., 1963; Galasko, 1981; Galasko, 1981; Ingall, 1964). Analogous "topical" approaches have been pursued more recently by groups specifically interested in the *in vivo* invasive properties of cancer cells in the bone (Koutsilieris et al., 1992b; Koutsilieris, 1992b; Nemoto et al., 1988b). However, these models are not suitable

for studies addressing other steps of metastasis, such as organ distribution, tumor cell arrest or tumor kinetics at multiorgan sites.

For these reasons, recent investigations have used either "experimental" or "spontaneous" models of bone metastasis. Experimental models are those in which cancer cells are injected into a blood vessel, where the choice of vessel will largely determine their organ distribution. In contrast, in spontaneous models, cancer cells are injected into soft tissue or an orthotopic organ site; tumors will then grow and "spontaneously" metastasize to different organs. The latter models are most attractive since they more closely mimic the behaviour and spread of cancer in clinical situations.

As detailed in Table 1, experimental bone metastasis were first reported by Coman and DeLong (Coman et al., 1951). Most groups have used immunocompetent rodents for metastasis with syngeneic cancer cells, and immunodeficient animals for studies with human cancer cell lines. Tumor cell lines have included epithelial tumors from prostate, breast, lung and cervix, malignant melanomas, neuroblastomas and sarcomas. Our group has reported spontaneous skeletal metastasis after intramuscular inoculation of tumor cells (Kostenuik et al., 1992). Others have relied on injection of cell suspensions into the blood stream. In these instances, bone involvement greatly depends upon the blood vessel used for inoculation. It is important to realize then, that due to the diversity of models

available, extrapolation of findings to general concepts on bone metastasis must be done cautiously. Although mechanisms may be similar in the different models, it is presumed that tumor-bone interactions are largely dependent on the cell type and host used.

TABLE 1. ANIMAL MODELS OF EXPERIMENTAL AND SPONTANEOUS BONE METASTASIS

Authors	Animals	Cells	Inoculation	Metastatic Distribution
Haq *et al.*, 1992	Copenhagen rats	Rat prostate Dunning R3327-Mat-LyLu	Intraarterial	Vertebrae
Kostenuik *et al.*, 1992	Fischer rats	Rat Walker 256	Intramuscular	Femurs
Arguello *et al.*, 1991	C57Bl/6 mice	Mouse Melanoma B16-G3.26, G3.5 and G3.15	Intraarterial	Multiostotic
	BALB/c mice	Mouse mammary tumors and lung carcinoma Line-1	Intraarterial	Multiostotic
	C3H mice	Mouse sarcoma KHT and RIF	Intraarterial	Multiostotic
	Athymic BALB/c nu/nu mice	Human cervical carcinoma HeLa, neuroblastoma SK-N-MC and sarcoma SKsarc	Intraarterial	Multiostotic

TABLE 1. ANIMAL MODELS OF EXPERIMENTAL AND SPONTANEOUS BONE METASTASIS (Cont.)

Authors	Animals	Cells	Inoculation	Metastatic Distribution
Shevrin *et al.*, 1988	Athymic NCr-nu/nu mice	Human prostate PC-3	Tail vein with vena cava occlusion	Vertebrae
Wang and Stearns, 1991	SCID mice	Human prostate PC-3 ML, MR, MC and MK	Intravenous	Lumbar vertebrae, ribs, cheek and knee, respectively
Geldof and Rao, 1990	Copenhagen rats	Rat prostate Dunning R3327-MatLyLu	Tail vein with vena cava occlusion	Vertebrae
Kjonniksen *et al.*, 1990	Athymic Han:rnu/rnu Rowett rats	Human melanoma LOX	Intraarterial	Multiostotic
Murphy *et al.*, 1988	Lister rats	Rat sarcoma MC28	Intraarterial	Multiostotic
Powles *et al.*, 1973	Wistar rats	Rat Walker 256	Intraaortic	Femurs and Tibiae
Coman and deLong, 1951	Long-Evans rats; rabbits	Rat Walker 256	Femoral with external abdominal pressure	Vertebrae

Bones are metabolically active structures. In particular, the mineralized matrix exhibits constant remodelling which requires active regulation by osteoblasts and osteoclasts. This process is tightly controlled by soluble regulatory peptides such as transforming growth factor β, platelet-derived growth factor, fibroblast growth factors, insulin-like growth factors and others (Mundy et al., 1990; Mundy, 1990; Canalis et al., 1989). These bone-related growth factors can be present as constituents of the extracellular matrix and are synthesized and secreted by bone cells. Although they are believed to have physiological roles within the bone microenvironment, in most instances their functions have not been elucidated. In general, they are thought to locally regulate the skeletal mass by modulating bone formation and resorption.

Four cancer phenotype attributes have been associated with the metastatic behavior of cancer cells in bone: (a) tumor cell growth, (b) tumor-induced osteolysis, (c) tumor cell adhesion to extracellular matrix components (Magro et al., 1985), and (d) organ-induced chemoattraction (Orr et al., 1990; Orr et al., 1982). Regarding tumor cell growth, extensive evidence has accumulated on the regulatory effects of different peptides in tumor cell growth. In particular, factors derived from resorbing bone have been shown to stimulate the proliferation of Walker 256 cells, an experimental rat bone-metastasizing cell line (Manishen et al., 1986). More specifically, this effect is inhibited by anti-TGF-β antibodies, supporting the observation that purified TGF-β in association with EGF stimulate Walker 256

cell proliferation *in vitro* (Millar-Book et al., 1990). This constitutes an exciting observation since bone is the organ with the largest amount of TGF-β in the body (Roberts et al., 1988). Paradoxically, TGF-β is well known for its growth inhibitory effects in most tumor cell lines, including human PC-3 prostate carcinoma cells (Goldstein et al., 1991).

Some investigators have explored the notion that growth factors from the bone marrow stimulate cancer cell growth. In effect, hematopoietic factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte CSF (G-CSF) and interleukin-3 (IL-3), have been shown to induce clonal growth of human colon adenocarcinoma cell lines (Berdel et al., 1989). Interestingly, increased growth of various tumor cell lines such as human LNCaP prostate carcinoma (Gleave et al., 1991), human lung carcinoma, mesothelioma and melanoma (Strobel et al., 1989), and B16 murine melanoma (Arguello et al., 1990) has been induced by conditioned media from stromal cells of human bone marrow. In reference to the cell lines of interest for this project, Chackal-Roy *et al.* demonstrated that factors derived from human bone marrow stromal cells stimulated the growth *in vitro* of PC-3 and DU-145 prostate cancer cell lines (Chackal-Roy et al., 1989). The factor responsible for this stimulation has been identified as transferrin (Chackal-Roy et al., 1991).

The work summarized in the first manuscript described initial observations on a murine model of bone metastasis with different cell lines and addressed the question of potential *in vivo* and *in vitro* correlations regarding tumor cell proliferation in the presence of bone-derived factors. Briefly, I asked if there was an association between the growth behavior of tumor cells *in vitro* after exposure to bone-derived growth factors and their bone metastatic pattern *in vivo*. For this purpose, I compared the *in vitro* and *in vivo* uptake of ^{3}H-thymidine (Simpson-Herren, 1987) and tumor burden in bone of the three murine tumor cell lines. Previous work had shown that FS/L10 cells were not stimulated to proliferate by conditioned bone culture media (Manishen et al., 1986).

Comparative *in vivo* and *in vitro* proliferation of murine tumor cells in the bone microenvironment

OTTO H. SANCHEZ-SWEATMAN[1], F. WILLIAM ORR[2] and GURMIT SINGH[1]

[1] Oncology Research Group, Room 4-02, Hamilton Regional Cancer Centre, McMaster University, 699 Concession Street, Hamilton, Ontario, Canada L8V 5C2
[2] Department of Pathology, University of Manitoba, Health Sciences Centre, 820 Sherbrook Street, Winnipeg, Manitoba, Canada R3A 1R9

Correspondence to: Dr. Gurmit Singh, Director of Research, Hamilton Regional Cancer Centre, McMaster University, 699 Concession Street, Hamilton, Ontario, Canada L8V 5C2.

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Abstract. To examine the effect of bone microenvironmental factors on the growth of metastatic cells, the *in vivo* proliferative features of three murine cell lines were determined at skeletal metastatic sites and correlated with their ability to grow *in vitro* in the presence of bone-derived factors. Bones, ovaries, adrenals and the brain were most affected by metastasis, following an intraarterial injection of B16/F1 and B16/F10 melanoma and FS/L10 fibrosarcoma cells into C57BL/6 mice. Melanoma cells showed a marked metastatic preference for bone, while fibrosarcoma cells developed brain metastasis in all animals. Tumor burden in bones was highest (19 ± 2%) for B16/F10 cells, compared to B16/F1 (10 ± 2%) or FS/L10 (3 ± 1%) cells. Autoradiographic studies demonstrated organ- and cell type-specific differences in tumor cell proliferation, with B16/F10 cells displaying the lowest labelling indexes in bone (12 ± 2% for B16/F10 *vs.* 28 ± 2% and 27 ± 4% for B16/F1 and FS/L10 cells, respectively). To test if bone-derived factors differentially affected tumor cell growth in these three cell lines, ^{3}H-thymidine uptake by these tumor cells was assessed after *in vitro* incubation with bone-derived conditioned medium. Under these conditions, we observed stimulation of B16/F10 cell proliferation, but inhibition of uptake in the other two cell lines. Thus, these results demonstrate that, in this *in vivo* experimental model, growth properties of metastatic cells are organ- and cell type-specific. Additionally, we show that the *in vitro* proliferative behavior of tumor cells in the presence of bone-derived factors correlates and may predict skeletal tumor growth properties *in vivo*.

Introduction

Prostate and breast cancers are two of the most common malignancies and display a metastatic preference for bone (1). The skeleton is most affected by secondary tumor lesions, constituting a significant cause of morbidity in cancer patients (2). Despite the obvious clinical relevance of bone metastasis, its pathogenesis has not been fully elucidated (3). Research in this area has recently concentrated on the interactions between bone-metastasizing tumor cells and the osseous microenvironment (4). Many of these studies have been conducted *in vitro* (5). These studies have suggested involvement of a number of mechanisms. In particular, enhanced growth, chemotaxis, and adhesion of cancer cells have been induced *in vitro* and *in vivo* by products released by bone marrow stromal cells (6-8), hematopoietic cells (9) and factors associated with bone resorption (10-13).

We have demonstrated the ability of conditioned medium from organ cultures of resorbing bones and transforming growth factor β to stimulate the growth of various human and experimental cancer cells *in vitro* (11,12). We postulate that the growth responses of experimental cancer cells *in vitro* correlate with their growth rates *in vivo* at metastatic foci in bone. In this study, we examined the metastatic behavior of three murine cancer cell lines following intraarterial injection, with respect to their tumor burden, proliferation *in vivo* at the metastatic sites, and proliferation in response to bone-derived conditioned medium. This is the first study

that demonstrates correlations between *in vitro* cancer cell proliferation by bone-derived conditioned medium and *in vivo* proliferation in bone.

Materials and methods

Cell lines. Murine melanoma B16/F1 cells, and their highly metastatic variant, B16/F10 (14), were cultured as monolayers with Dulbecco's modified Eagle's medium at 37°C in a 5% CO_2 air atmosphere. Similarly, FS/L10 cells, derived from a murine methylcholanthrene-induced fibrosarcoma (15), were cultured in RPMI-1640 culture medium. Media were supplemented with 10% fetal bovine serum, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulphate, 0.25 μg/ml amphotericin B and 73 μg/ml L-glutamine. These three cell lines are all syngeneic with C57BL/6 mice.

Intraarterial injection and follow-up. Injections of cancer cells into the left ventricle were performed by a modified procedure as described by Arguello *et al.* (16). Briefly, subconfluent cells were harvested with 0.01 M ethylenediamine-tetraacetic acid (EDTA) (GIBCO Laboratories, Grand Island, NY), resuspended and rinsed in Hanks' balanced salt solution, pH 7.4, to assure complete removal of bovine serum from the inoculate. After disaggregating the cells by repeated passes

through an 18-gauge needle, cell viability was determined by trypan blue exclusion. Cell suspensions with viability below 95% were not used.

Three groups of 10 female inbred C57BL/6 mice (Charles River, Montreal, QB), 10 weeks of age and weighing 20-30 g were used. Each mouse was anesthetized by an intramuscular injection of 32 mg/kg ketamine hydrochloride (Rogarsetic™, Rogar/STB, London, ON) and 32 mg/kg xylazine hydrochloride (AnaSed™, Lloyd Laboratories, Shenandoah, IO). After performing a left parasternal longitudinal midline incision of the skin, a 30-gauge needle was inserted through the second intercostal space, until bright pulsating blood was observed in the catheter. At this stage, 0.1 ml of cell suspension containing 10^5 cells was injected within 10-20 seconds. After removing the needle, the incised skin was sutured and the animals were allowed to recover.

Animals were observed daily for signs of weakness or hind leg paralysis. As soon as either of these signs was observed, the animals in the group received 100 μCi ^{3}H-thymidine intraperitoneally. Autopsies were performed and all viscera macroscopically examined after a 24-hour fixation in phosphate-buffered formaldehyde, pH 7.0. Samples processed for light microscopy included tissues from brain, heart, lungs, liver, spleen, pancreas, kidneys, ovaries and any macroscopically suspicious lesions. The left tibia (proximal third), left femur (distal third), left humerus (proximal third), all lumbar vertebrae, and both maxillae and

mandible were dissected, decalcified with Perenyi's fluid (17) for 24 hours and processed for light microscopy.

Determination of tumor burden. Histological slides prepared from bones and other organs were analyzed to establish the proportional area of tumor burden. The total area of each tissue section and the area involved by tumor in each section was determined with a digitizing pad (Digi-Pad™, GTCO, Rockville, MD). Tumor burden was then expressed as a percentage of the total area of the organ compromised by metastatic tumor. For each animal, up to 6 bone sections and up to 2 sections of other organs were analyzed.

In vivo assessment of tumor proliferation. Histological sections containing metastatic lesions were processed for autoradiography. For this purpose, sections were dewaxed with xylene and hydrated through decreasing concentrations of ethanol. They were then immersed into pre-warmed photographic emulsion (NTB2, Kodak, Rochester, NY), air dried, and kept in the dark at 4°C. After 3 weeks, slides were developed with fresh D-19 High Contrast Negative Developer, fixed with Kodak Fixer, stained with hematoxylin-eosin and mounted. The number of grains *per* nucleus was counted in 100 randomly selected cells of each metastatic lesion. The labelling index was defined as the proportion of cancer cells in which the nucleus contained 10 or more grains.

Preparation of bone-derived conditioned medium (BDCM). The method for organ culture of resorbing bones was adapted from Manishen *et al.* (11). In brief, three pregnant C57BL/6 mice were each injected subcutaneously with 10 µCi of ^{45}Ca on day 15-16 of gestation. On the second day after delivery, the calvariae were aseptically prepared from newborn mice. In order to remove any unincorporated ^{45}Ca, each parietal bone was placed into a culture well containing serum-free Dulbecco's modified Eagle's medium or RPMI-1640 and incubated for 24 hours on an orbital shaker at 37°C in a 5% CO_2. Bone resorption was induced by replacing the serum-free medium with 2 ml of medium supplemented with 10% fetal bovine serum and antimicrobial agents as described above. Three days later, bone-derived conditioned medium was recovered, vacuum-filtered through a 0.2-µm filter (Sterilization Filter Unit Type S, Nalge, Rochester, NY) and aliquoted for further use. Bone resorption was calculated from ^{45}Ca release as follows:

$$^{45}\text{Ca release} = \frac{\sum \text{cpm [BDCM samples]}}{\sum \text{cpm [BDCM samples]} + \sum \text{cpm [Bone samples]}}$$

For the present experiments, ^{45}Ca release was calculated to 24±5% for RPMI-BDCM and 28±7% for Dulbecco's modified Eagle's medium-BDCM.

In vitro assessment of tumor cell proliferation in the presence of bone-derived conditioned medium. All three cell lines were harvested with 0.01 mM EDTA, resuspended in serum-supplemented medium and disaggregated by repeated passage through an 18-gauge needle. Cell viabilities were determined by trypan blue exclusion. Cells were seeded into 96-multiwell plates at a concentration of 2,500 cells/well. Each cell line was exposed to increasing concentrations of bone-derived conditioned medium (0%, 20%, 40% and 80%), diluted in serum-supplemented Dulbecco's modified Eagle's medium or RPMI to total volumes of 250 µl. Each BDCM concentration was tested in 6 individual wells. After incubation periods of 24, 48 and 72 hours at 37°C in a 5% CO_2 air atmosphere, cell proliferation was determined by ^{3}H-thymidine incorporation as described below.

To determine proliferative activity, 1 µCi ^{3}H-thymidine was added to each culture well and cells were incubated for 2 hours at 37°C in a 5% CO_2 air atmosphere. The medium with unincorporated label was then removed and 100 μl of a 10x solution of trypsin/EDTA were added to each well and left for 1 hour to obtain complete detachment of monolayers. Then, 150 μl of cold (4°C) 20% trichloroacetic acid in PBS pH 7.2 was added and left overnight. Acid-precipitated material was then filtered with a PHD™ Cell Harvester (Cambridge Technology, Watertown, MA) onto glass fiber filter discs (Glass Fiber Filter Strips 240-1, Cambridge Technology, Watertown, MA). Discs were then immersed into 3-ml

scintillation fluid (Ecolite™, ICN Biomedicals, Irvine, CA) and radioactivity measured by liquid scintillation in a beta counter (LKB Wallac 1209 Rackbeta, Turku, Finland).

Statistics. The statistical significance of differences was determined by unpaired Student's *t* test.

Results

Metastatic distribution after intraarterial injection. Latency period, defined as the time elapsed between tumor cell inoculation and death of one member of the group, was 14 days for animals injected with B16/F1 cells, 17 days for B16/F10 and 18 days for FS/L10. On microscopical examination, 7/10 mice for B16/F1, 7/10 for B16/F10 and 8/10 for FS/L10 had evidence of metastatic lesions in the viscerae and/or skeleton. As observed in Table I, the organs most frequently displaying metastatic lesions were bones, ovaries and adrenals. Brain metastasis were observed in all animals injected with FS/L10 cells. Among the bones studied, the most affected were the maxilla, followed by mandible, femur, tibia, vertebrae and humerus (Table II).

Skeletal and visceral tumor burden. As a group (Table IIIA) or individually (Table IIIB), tumor burden in the bones was highest in mice injected with B16/F10 cells, when compared with the two other cell lines, B16/F1 and FS/L10 ($P<0.002$ and $P<0.0001$, respectively). In general, skeletal metastases by the three cell lines showed a relatively low tumor burden: other metastatic sites such as ovaries and adrenals displayed the highest percentage area occupied by tumor. The latter results are explained on the basis that ovaries and adrenals are small organs which were histologically examined entirely and are promptly replaced by metastatic nodules. In contrast, the histological sections of bone and other organs examined constituted representative tissue samples.

In vivo proliferation at metastatic sites. Autoradiographic determination of proliferative labelling indexes for metastases in bones and other organs are shown in Table IV. For the three cell lines, the proportion of metastatic cells undergoing proliferation differed among organs: bones for B16/F1 cells ($P<0.02$ *vs.* ovaries and brain), ovaries for B16/F10 cells ($P<0.0001$ *vs.* bones) and adrenals for FS/L10 cells ($P<0.001$ *vs.* ovaries) displayed the highest proliferation rates when compared with other metastatic sites. These results indicate that dissimilar cancer cells proliferate differently in individual organs. Despite the high tumor burden displayed by B16/F10 cells in bones (see above), their tumor cell proliferation in skeletal metastases was more than 50% lower than those for ovarian and adrenal tumors ($P<0.0001$).

In vitro proliferation in the presence of bone-derived factors. Under basal conditions the three cell lines doubled within the first 24 hours: 19 hours for B16/F1, 21 hours for B16/F10 and 14 hours for FS/L10. The bone-derived conditioned medium used for this study stimulated growth of rat mammary Walker 256 cells, as previously described (12). When B16/F10 cells were exposed to increasing concentrations of bone-derived conditioned medium for 24 hours, ^{3}H-thymidine uptake was 1.7-fold higher than control with up to 40% bone-derived conditioned medium (P<0.03) (Figure 1). All other cells displayed concentration-dependent inhibition of proliferation.

Correlation between in vivo and in vitro proliferative properties. When comparing the *in vivo* proliferative parameters of bone metastases among the three cell lines studied, there was a noticeable contrast between B16/F10 cells and the other two cell lines (Table V): although B16/F10 cells displayed the highest skeletal tumor burden of all three cell lines, they showed the lowest labelling indexes in bone, relative to B16/F1 and FS/L10 cells. Furthermore, this same cell line was the only one in which *in vitro* growth stimulation by bone-derived conditioned medium was observed (Figure 1 and Table V). The correlation coefficients (r) between *in vitro* proliferation in the presence of bone-derived conditioned medium and the *in vivo* parameters for the three cell lines were 0.970 for bone tumor burden and -0.974 for labelling indexes at skeletal metastases.

Discussion

Bone metastases are clinically and morphologically associated with active local bone remodelling (18,19). Various growth factors critical for the local regulation of bone metabolism such as transforming growth factor ß and others, have been shown to modify phenotypic characteristics of malignant cells such as invasiveness, attachment, growth and migration (20-27). Moreover, the availability and activity of these regulatory peptides in bone are dependent on the local metabolic status which can be directly or indirectly modified by the cancer cells (28). These observations have led us to postulate that growth factors relevant for the metabolism of bone modify certain malignant features of metastatic cancer cells, and indeed, may locally promote the development of metastasis. In this study we investigated whether the local factors associated with bone resorption can stimulate proliferation of murine cancer cells. Using three murine tumor cell lines with different metastatic behavior, we determined growth and proliferation parameters in bone metastases *in vivo* and *in vitro* in the presence of bone-derived conditioned medium obtained from actively resorbing mouse fetal calvariae. This medium has been shown to contain transforming growth factor β and other growth factor activities (12,29).

Following left ventricular injection of cancer cells, the organs most affected by metastatic lesions were the bones, adrenal glands and ovaries. This distribution was independent of tumor cell type. However, FS/L10 cells displayed a well-defined neurotropism: all animals with this tumor had metastatic lesions in the central nervous system. The distribution of skeletal secondary tumors predominantly affecting maxilla, mandible, femur and tibia was similar to that described in other experimental animals after intraarterial injection (16,30,31) and in human clinical cases (32,33). The fact that there were no metastases in the kidneys, supports the notion that a good arterial supply and dense capillary network are not sufficient for the development of secondary lesions. The local organ microenvironment is important in maintaining cancer cell viability and behavior following vascular arrest.

The *in vivo* proliferative properties of the three murine cell lines were independent of the cell lineage. These cell type- and site-dependent variations in tumor cell proliferation support the idea that the growth of metastatic cells is affected by organ-specific microenvironmental factors. The differences observed in skeletal and visceral tumor burden, and proliferative labelling indexes between B16/F1, B16/F10 and FS/L10 cells also support a differential proliferative response of metastatic cancer cells to local tissue factors (34). Evaluation of skeletal tumor burden by B16/F10 cells showed that they displayed the highest bone involvement. However, B16/F10 cells had a lower labelling index in bone compared to the other two cell lines.

These *in vivo* observations were evaluated *in vitro*, following exposure of the tumor cell lines to factors associated with bone resorption, by using the same proliferative marker as employed *in vivo*. Only B16/F10 cells showed an increase in ^{3}H-thymidine uptake when exposed to bone-derived conditioned medium. As stated above, B16/F10 cells displayed *in vivo* the lowest ^{3}H-thymidine uptake in bone. Metastatic lesions of B16/F10 cells in bone were, in contrast, the largest when compared with other cell lines. We found a strong correlation between ^{3}H-thymidine uptake *in vitro* in the presence of BDCM and *in vivo* tumor burden. However, a negative correlation between *in vitro* and *in vivo* proliferation was observed. Thus, our data support an association between the *in vitro* and *in vivo* kinetics of tumor cell populations when exposed to local factors in the bone microenvironment. In this study we examined two kinetic parameters that are quantifiable and represent tumor cell proliferation and growth. It is postulated that B16/F10 cells, upon arrest in bones, initially proliferate rapidly due to the presence of bone-derived factors. The growth is hindered by hypoxia or other local limiting factors, such as exhaustion of bone factors.

In conclusion, we have demonstrated that a relationship exists between the *in vivo* and *in vitro* behavior of metastatic cancer cells. Further investigation of host-tumor cell interactions, and the effects of the bone microenvironment, will lead to a better understanding of the pathogenesis of bone metastasis. This will eventually provide therapeutic advances against human skeletal metastasis.

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Table I. **Metastatic distribution after intraarterial injection of murine tumor cell lines in C57BL/6 mice**

Organs	B16/F1 n=7*	B16/F10 n=7*	FS/L10 n=8*
	Percentage of animals with metastases		
Bones	100	86	75
Ovaries	100	100	38
Adrenals	71	86	88
Brain	43	29	100
Lungs	0	14	63
Pancreas	0	0	38
Kidneys	0	0	0

* Left ventricular injection of 10^5 cancer cells/mouse was performed following a procedure described by Arguello *et al.* (16). All viscerae were microscopically examined to determine the presence of metastatic lesions. **n** represents the number of animals in which metastatic lesions developed.

Table II. **Distribution of bone metastases after intraarterial injection of murine tumor cell lines in C57BL/6 mice**

Bones	Percentage of bones with metastasis		
	B16/F1 n=7*	B16/F10 n=7*	FS/L10 n=8*
Maxilla	100	86	75
Mandible	100	86	50
Femur	100	57	0
Tibia	100	57	0
Vertebrae	71	71	0
Humerus	43	57	13

* Left ventricular injection of 10^5 cancer cells/mouse was performed following a procedure described by Arguello *et al.* (16). Microscopical examination of selected bones was performed in decalcified, paraffin-embedded sections. **n** represents the number of animals in which visceral metastatic lesions developed.

Table IIIA. Visceral metastatic tumor burden after intraarterial injection of murine tumor cell lines in C57BL/6 mice*

	B16/F1		B16/F10		FS/L10	
Organs	**n**	**Mean ± SEM**	**n**	**Mean ± SEM**	**n**	**Mean ± SEM**
Bones	7	10 ± 2%	6	19 ± 2%	6	3 ± 1%
Ovaries	7	53 ± 12%	7	85 ± 4%	3	39 ± 18%
Adrenals	5	11 ± 4%	6	58 ± 7%	7	35 ± 7%
Brain	3	1 ± 1%	2	6 ± 6%	8	1 ± 0%
Lungs	0	—	1	1%	4	3 ± 1%
Pancreas	0	—	0	—	1	2%
Kidneys	0	—	0	—	0	—

Table IIIB. Skeletal metastatic tumor burden after intraarterial injection of murine tumor cell lines in C57BL/6 mice*

	B16/F1		B16/F10		FS/L10	
Bones	**n**	**Mean ± SEM**	**n**	**Mean ± SEM**	**n**	**Mean ± SEM**
Femur	5	15 ± 3%	4	22 ± 8%	0	—
Tibia	7	14 ± 4%	4	28 ± 5%	0	—
Humerus	2	12 ± 7%	4	26 ± 2%	0	—
Vertebra	3	6 ± 2%	5	13 ± 3%	0	—
Mandible	5	9 ± 1%	6	27 ± 2%	4	5 ± 2%
Maxilla	4	3 ± 0%	5	3 ± 1%	6	3 ± 1%

* Tumor burden was determined in histological sections of the bones by establishing the total area of each tissue section and the proportion of this area displaying tumor. Tumor burden is thus expressed as a percentage of the total area in the bone section compromised by metastatic tumor. **n** represents the number of animals in which metastatic lesions were found in specific organs (IIIA) and bone sites (IIIB).

Table IV. Tumor cell proliferation in skeletal and visceral metastasis after intraarterial injection of murine tumor cell lines in C57BL/6 mice

	B16/F1		B16/F10		FS/L10	
Organs	**n**	**Labelling Indexes**	**n**	**Labelling Indexes**	**n**	**Labelling Indexes**
Bones	7	28 ± 2%	5	12 ± 2%	6	27 ± 4%
Ovaries	7	21 ± 4%	6	30 ± 2%	3	4 ± 1%
Adrenals	2	19 ± 3%	5	28 ± 2%	7	39 ± 5%
Brain	2	21 ± 1%	1	8%	7	36 ± 3%
Lungs	0	---	0	---	5	35 ± 5%
Pancreas	0	---	0	---	3	34 ± 8%
Kidneys	0	---	0	---	0	---

Each mouse received 100 μCi ^{3}H-thymidine intraperitoneally two hours before death. Histological sections containing metastatic lesions were processed for autoradiography. Labelling indexes were determined by calculating the proportion of cancer cells in which nuclei contained 10 or more grains, and are thus expressed as the percentage mean ± SEM from 100 randomly selected tumor cells.

Table V. **Correlation between *in vivo* and *in vitro* proliferative behavior of three murine cancer cell lines**

Cell lines	Skeletal tumor burden	Skeletal labelling index[a]	*In vitro* proliferation in the presence of bone-derived factors[b,c]
B16/F1	10 ± 2%	28 ± 2%	49 ± 15%
B16/F10	19 ± 2%	12 ± 2%	147 ± 27%
FS/L10	3 ± 1%	27 ± 4%	34%

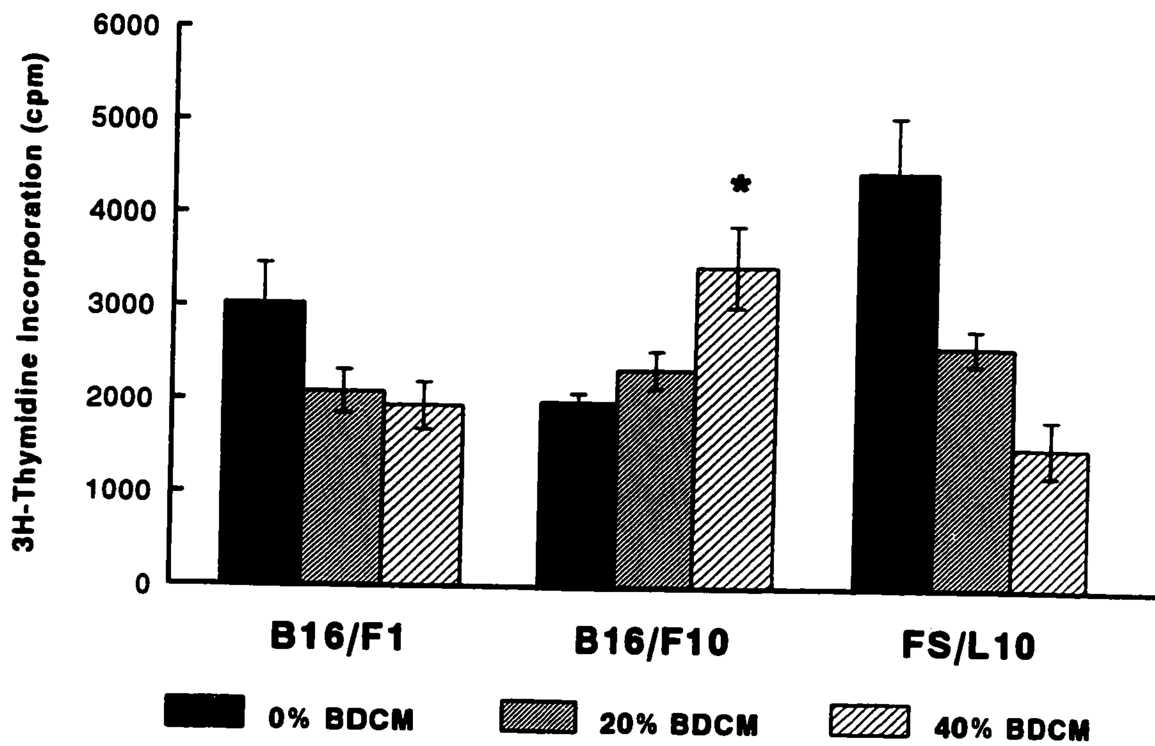
[a] Expressed as percentage of cancer cells with 10 or more grains *per* nucleus.

[b] Expressed as percent ± SEM of ^{3}H-thymidine uptake in control (0% BDCM for 24 hours).

[c] Correlation (*r*) for values of skeletal tumor burden and *in vitro* proliferation data is 0.970; *r* value for skeletal labelling index and *in vitro* proliferation data is -0.974.

Figure Legend

Figure 1. *In vitro* proliferation of murine tumor cells in the presence of bone-derived conditioned medium. Cell lines were exposed *in vitro* to increasing concentrations (0-40%) of bone-derived conditioned medium (BDCM). After an incubation period of 24 hours, cell proliferation was determined by ^{3}H-thymidine incorporation. Only B16/F10 cells showed increased ^{3}H-thymidine uptake in the presence of increasing concentrations of bone-derived conditioned medium (asterisk; $P<0.03$). Proliferation of the other two cell lines was inhibited by similar conditions. Each bar represents the mean ± SEM of 6 individual replicates.



3H-Thymidine Incorporation (cpm)
6000
5000
4000
3000
2000
1000
0
*
B16/F1
B16/F10
FS/L10
0% BDCM
20% BDCM
40% BDCM

3. Additional Results and Discussion

The original studies using this animal model allowed me to make two additional observations. First, it was obvious that the technical aspects were challenging and that the success rate of injections into the left ventricle and metastasis formation was not 100%: about 20% (5/26) of animals would die during or immediately following the procedure, often due to hemopericardium. Second, radiological analysis of the skeleton in animals injected was not sensitive enough to detect bone metastasis by murine B16/F10 melanoma cells: the technique queried, but did not assert, the presence of bone lesions in 71% (5/7) of animals with bone metastasis. Furthermore, there were 93% (13/14) of animals diagnosed as false positives and 14% (1/7) as false negatives. When considering each bone individually, the sensitivity of radiology was 25% (7/28) with 16% (32/200) false positives. As a consequence of these results, radiological analysis was not used to follow-up metastatic development *in vivo*.

In vivo studies on tumor cell proliferation led to technical developments: ^{3}H-thymidine and bromodeoxyuridine (BrdU) incorporation techniques were considered as alternatives for the assessment of tumor cell proliferation in bone metastasis (Lacy et al., 1991; Eldridge et al., 1990). To guarantee immunodetection of BrdU in bone sections, two fixation protocols were compared, namely, formaldehyde and ethanol, with non-decalcified and decalcified bones. BrdU staining was successfully

detected in decalcified bones. Moreover, immunohistochemical detection was accomplished in ethanol-fixed tissues, but not in formaldehyde-fixed bones. In contrast, plastic-embedding of bones did not provide, in my hands, consistent BrdU labelling.

The assessment of tumor burden in different organs with the Merz graticule has the limitation that it is determined as a percentage of the total organ area. Thus, when the organ is small, a small tumor nodule will occupy almost 100% of the organ. In contrast, the same small nodule, in a large organ, such as the brain, will occupy a small percentage of this organ. Thus, we were cautious in arriving to any conclusions based on this methodological bias. It is worth noting here that the data obtained from this *in vivo* work were collected by one observer who was not aware, at the time of collecting the data, of the specific conditions used on each animal.

The metastatic distribution in this animal model of experimental secondary tumors was predictable to a point: independently of the type of tumor cell injected, some organs were preferentially affected by metastatic lesions. These included the bones, ovaries, adrenals and brain. However, there were some organs that still, despite these features of the model, showed preferential "cell type-specific" metastatic distribution. A typical example of this was the high incidence of brain metastasis after injection with FS/L10 fibrosarcoma cells.

Additional *in vitro* studies were done to evaluate tumor cell proliferation in the presence of bone-derived factors. Specifically, aside from the murine tumor cell lines mentioned in the manuscript, human cell lines were tested including breast MCF-7, lung A-549 and colon HT-29 adenocarcinomas and SK-N-SH neuroblastoma. None of these cells showed a proliferative response in the presence of 20-80% bone-derived conditioned medium. In this context, there is no precedence in the literature for a response by these cells to bone-derived conditioned medium. However, growth stimulation by bone-derived factors has been demonstrated *in vitro* in human cell lines including prostate LNCaP (Gleave et al., 1991, 1992) and PC-3 adenocarcinoma (Chackal-Roy et al., 1989; Chackal Rossi and Zetter, 1992), and in primary cultures from human lung and breast cancers and malignant melanoma (Strobel et al., 1989). The lack of proliferative effect in the cells tested, may be due to a lack of specific growth-promoting activity in the medium used or a cytotoxic effect in these cells by this medium.

In summary, the initial experiments with the murine animal model emphasized the characterization of the metastatic distribution of three different murine cell lines after intraarterial injection. Additionally, an initial stress on the evaluation of tumor cell proliferation *in vivo* showed that this variable is different in bones when compared with that in other organ sites, and that it may be possible to correlate proliferative characteristics of tumor cells *in vivo* with their proliferative characteristics *in vitro*.

C. OSTEOLYSIS BY MURINE MELANOMA CELLS

1. Introduction

Studies in our laboratory used, for many years, as a model for a bone-metastasizing tumor cell phenotype, the Walker 256 cell line, originally isolated from a rat mammary tumor. Following subcutaneous injection into rats, Walker 256 cells displayed metastatic patterns preferentially involving bones and other organs such as lungs, liver and lymph nodes. This pattern developed within two weeks in association with fulminant hypercalcemia (Kostenuik et al., 1992b), thus constituting an optimal means of achieving spontaneous metastasis. Using this *in vivo* model, Kostenuik *et al.* demonstrated that stimulation of bone resorption by endogenous parathyroid hormone-related protein from non-metastasizing Rice H-500 Leydig tumors selectively induced Walker 256 cell growth in bone (Kostenuik et al., 1992a). In contrast, bisphosphonates, which inhibit bone resorption, increased trabecular bone volume and, surprisingly, also enhanced skeletal tumor burden and tumor cell proliferation (Kostenuik et al., 1993). Furthermore, *in vitro* work with these cells pointed towards enhanced proliferation and chemoattraction of Walker 256 cells by TGF-β, a product of bone resorption. Bone-derived conditioned medium, obtained from actively resorbing bone organ cultures, and thus containing TGF-β activity, not only increased the same properties, but also promoted adhesion of

Walker 256 cells to cultured bones (Magro et al., 1985; Orr et al., 1990; Millar-Book et al., 1990; Manishen et al., 1986).

The murine model of experimental bone metastasis initially described in 1988 (Arguello et al., 1988), had the advantage of enabling the use of different tumor cells to produce bone metastasis, including epithelial cell lines such as melanomas. The biology and potential interactions of these cells with bone are probably different from those of Walker 256 cells. Thus, I decided to perform histological analysis of the skeletal lesions by murine B16/F1 and B16/F10 cells. This showed that, in contrast to other models of bone metastasis, local bone destruction by these cells was not associated with osteoclastic proliferation, as determined by routine histology, and later by histochemical identification of osteoclast acid phosphatase activity. Rather, tumor cells seemed to be closely associated with bone surfaces undergoing pathological resorption. This observation lead this project into querying the possibility that cancer cells might be capable of degrading bone directly, without necessarily requiring osteoclasts or other bone cells to do so. As detailed in the following manuscript, we gathered *in vivo* and *in vitro* evidence demonstrating this to be the case. Participation of tumor-derived matrix metalloproteinases in this degradative process was also suggested by *in vitro* experiments. These findings are provocative since they challenge the traditional "dogma" that tumor cells can only induce bone degradation *via* osteoclast activation, and, in hindsight, have precedence in the scientific literature.

Direct Osteolysis Induced by Metastatic Murine Melanoma Cells: Role of Matrix Metalloproteinases

O.H. Sanchez-Sweatman[1], J. Lee[2], G. Singh[1], and F.W. Orr[2]

[1]Departments of Pathology and Oncology Research Group, McMaster University, Hamilton, Ontario; and [2]Department of Pathology, University of Manitoba, Winnipeg, Manitoba, Canada

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Name and address for correspondence:

Dr. Gurmit Singh
Research, Hamilton Regional Cancer Centre
699 Concession Street
Hamilton, Ontario
Canada L8V 5C2

Tel: (905) 387-9711 ext. 7007
FAX: (905) 575-6330

Running title:

Matrix Metalloproteinases in Bone Metastasis

We examined the osteolytic ability of metastatic cells and a role for tumour matrix metalloproteinases (MMPs) in bone degradation. Histomorphometry of experimental bone metastases by B16/F1 melanoma cells showed that osteolysis was associated with a 90% decrease in osteoclast number and predominance of cancer cells overlaying resorption pits. *In vitro*, B16/F1 cells and their conditioned medium (CM) degraded ^{3}H-proline-labelled extracellular matrices from osteoblast-like cells and ^{45}Ca-labelled calvariae. Using bone slices, we observed morphological evidence of degradation by B16/F1 cells. A role for tumour MMPs in bone degradation was supported by inhibition of degradation by 1,10-phenanthroline, collagen I degradation by tumour cells and presence of TPA-inducible M_r 90,000, 84,000 and 64,000 gelatinolytic, and 54,000 caseinolytic bands in B16/F1-CM. These studies indicate that metastatic cancer cells degrade bone matrix directly and that this is partially mediated by MMPs.

Key words: Metastasis, Bone, Matrix Metalloproteinases, Osteolysis, Melanoma.

INTRODUCTION

Skeletal metastases are a common problem in patients with cancer of the breast, prostate and other malignancies, presenting as osteolytic lesions with pain, fractures and hypercalcemia (1). Osteoclasts, tumour-associated macrophages and cancer cells have been identified as cellular mediators (2). Although the mechanisms by which osteolysis occurs have not been completely elucidated, osteoclast mediation has been extensively examined, especially regarding cancer-associated hypercalcemic syndromes (3). In these situations, cancer cells may activate osteoclasts by secreting hormones, growth factors or cytokines (4). Tumour-associated macrophages (5) and tumour cells can directly degrade bone matrix by releasing hydrolytic enzymes (6,7). However, the mechanisms by which these phenomena occur have not been elucidated.

Tumour-derived enzymes that degrade extracellular matrix have been described in the context of tumour invasion and metastasis. Type I collagen, the most predominant protein in bone matrix (8), is degraded by interstitial collagenases and gelatinase A (9). These enzymes belong to the family of matrix metalloproteinases (MMPs), are secreted by cells as proenzymes and activated by extracellular components including plasmin. Upon activation, and in the presence of divalent cations, these enzymes degrade their substrates.

We have used histomorphometry to demonstrate that osteolysis occurs in experimental bone metastases by murine melanoma cells. Our findings suggest that tumour cells directly degrade bone matrix. This study shows that B16/F1 cells degrade bone-related and mineralized bone matrices *in vitro*, and provides evidence that tumour-derived MMPs mediate this phenomenon.

MATERIALS AND METHODS

Intraarterial injection of B16/F1 cells

Murine melanoma B16/F1 cells (10) were cultured as monolayers in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulphate, at 37°C in 5% CO_2. Intraarterial injections followed procedures by Arguello *et al.* (11). Subconfluent cells were harvested with 0.01 M ethylenediamine-tetraacetic acid (EDTA) and rinsed in Hanks' balanced salt solution (pH 7.4) to assure removal of FBS. Ten-week-old inbred C57BL/6 female mice (Charles River, Montreal, PQ) were anaesthetized by intramuscular injection of 32 mg/kg ketamine hydrochloride (Rogarsetic™, Rogar/STB, London, ON) and 32 mg/kg xylazine hydrochloride (AnaSed™, Lloyd Laboratories, Shenandoah, IO). A left parasternal longitudinal skin incision was performed to locate the second intercostal space. A 30-gauge

needle was inserted through the thoracic wall until bright pulsating blood was observed in the catheter. 0.1 ml of suspension containing 10^5 cells was injected within 20 seconds and followed by skin suture. After animals showed hind leg paralysis, autopsies were performed and viscera examined macroscopically and microscopically. The left tibia (proximal third), left femur (distal third), left humerus (proximal third), lumbar vertebrae, maxillae and mandible were dissected, decalcified and processed for light microscopy.

Histomorphometry of bone metastases

Histological sections stained with haematoxylin and eosin were evaluated. The left distal femoral epiphysis of each mouse was selected for morphometric analysis, since it is a common site for metastases after intraarterial injection of melanoma cells (11). With a Merz eyepiece graticule (12), three different parameters were determined: (a) cross-sectional areas of bone, bone marrow, and tumour, expressed as percentages of total area examined, (b) proportions of cell subpopulations (osteoblasts, osteoclasts, tumour cells and resting cells) overlaying trabecular surfaces, expressed as percentages of total number of line intercepts, and (c) proportions of resorption pits overlaid by osteoclasts or cancer cells. Seven mice without bone lesions were used to determine histomorphometric parameters in control bones. These mice had been injected with B16/F1 cells in sites other than

the left ventricle, and did not have bone metastases as determined by histological examination of seven different long or flat bones.

Detection of tartrate-resistant acid phosphatase activity (TRAP) in bone sections

To assure preservation of enzymatic activity, femurs were fixed in 80% ethanol for at least 24 hours and plastic-embedded without decalcification. Staining for TRAP was accomplished by incubation of the sections at 37°C for 75 minutes with a solution containing acetate solution, naphthol AS-BI phosphoric acid and tartrate solution (Sigma Diagnostics, St. Louis, MO). This was followed by rinsing and counterstaining with acid haematoxylin solution. The number of osteoclasts in ten microscopic fields was then determined.

Degradation of osteosarcoma-derived extracellular matrices

Radiolabelled extracellular matrices were prepared by culturing human osteosarcoma Saos-2 and U-2 OS cells (ATCC, Rockville, MD) in Minimum Essential Medium supplemented with FBS and antibiotics, *plus* 5 μCi/ml ^{3}H-proline (NEN, Boston, MA) on 96-well plates for 5 days. Cells were lysed with 20 mM NH_4OH at 37°C for 20 minutes and the radiolabelled matrix remained at the bottom of the wells (13). After rinsing with phosphate-buffered saline (PBS), matrices were incubated with 10^5 B16/F1 cells *per* well or 200 μl fresh conditioned medium (CM)

per well. This conditioned medium was collected by incubating subconfluent B16/F1 monolayers in Minimum Essential Medium under serum-free conditions for 24 hours. Release of ^{3}H was determined 24 hours later by collecting 50 μl aliquots of medium into scintillation fluid (Ready Safe™, Beckman, Fullerton, CA) and counted in a Beckman LS1801 liquid scintillation beta counter (Beckman, Irvine, CA). Each value was expressed as the mean ± SEM of 8 replicates. Mineralization of Saos-2 matrices was obtained by incubation with 10 mM β-glycerophosphate (Sigma, St. Louis, MO) for 2 weeks (14). Crystal formation was evident with Saos-2 but not U-2 OS cells. Dependence of matrix degradation on divalent cations was determined by adding 0-50 mM 1,10-phenanthroline (Sigma, St. Louis, MO) to the samples of B16/F1-CM prior to the degradation assay.

Degradation of rat fetal calvariae

To prepare radiolabelled calvariae, pregnant Sprague-Dawley rats were injected subcutaneously with 10 μCi ^{45}Ca on day 16 of gestation (15). On day 20, fetal calvariae were dissected, immersed in serum-free Minimum Essential Medium and frozen at -20°C to induce death of bone cells. After 24 hours, calvariae were thawed, rinsed and incubated with 10^{5} B16/F1 cells or 1 ml fresh B16/F1-CM medium *per* bone for 24 hours at 37°C. ^{45}Ca release was determined as described above. Each value was expressed as the mean ± SEM of 4 replicates.

Resorption pit assay

Longitudinal bovine cortical bone slices from the femoral midshaft were prepared according to Chambers *et al.* (16). Slices (5x5x2 mm) were cut with an Isomet low-speed diamond-edged saw (Buehler Instruments, Evanston, IL), cleaned by ultrasonication for 30 minutes, washed in ethanol, dried, and stored. After the slices were presoaked with serum-free medium, 10^5 B16/F1 cells were placed onto the slices and incubated for 60 min at 37°C to allow cell attachment. Non-adherent cells were removed by rinsing with serum-free medium. Incubation in 5 ml MEM with 10% FBS and antibiotics was allowed at 37°C for 3-7 days. After cancer cells were removed with 0.1% Triton X-100 in dH_2O for 6 hours, fixation of the slices with 10% formaldehyde or 2.5% glutaraldehyde in PBS was performed. Slices were stained with 1% toluidine blue for light microscopy according to Ritchie *et al.* (17), or prepared for scanning electron microscopy by ethanol dehydration, critical point drying and gold sputter-coating.

Degradation of type I collagen

Purified rat type I N-[propionate-2,3-^{3}H]propionylated-collagen (DuPont, Markham, ON) was dissolved in 0.01 N acetic acid and placed on plastic wells at a concentration of 5 $\mu g/cm^2$ at 37°C. After 24 hours, the wells were rinsed with PBS

and exposed to 10^5 B16/F1 cells or 200 μl fresh CM *per* well for 24 hours at 37°C. Release of ^{3}H into the medium was measured as described.

Enzymography of B16/F1-derived CM in gelatin- and casein-embedded gels

Cancer cells were cultured to near-confluency in serum-supplemented Minimum Essential Medium. Rinsing with PBS was followed by incubation with serum-free MEM and antibiotics with 0-400 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 24 hours. Samples of B16/F1 conditioned medium were collected and centrifuged to remove cell debris. Protein-standardized samples were resolved at 4°C in 10% SDS-PAGE containing 1 mg/ml gelatin or casein (Sigma, St. Louis, MO) (18). Incubation with 2.5% Triton X-100 for 30 minutes was followed by enzymatic degradation of the substrate in a buffer containing 0.1 M Tris-HCl (pH 8.0), 5 mM $CaCl_2$ and 0.04% NaN_3, for 48 hours at 37°C. Gels were stained with 2.5% Coomassie Blue for 30 minutes, and destained with methanol/acetic acid/water (40:10:50). Densitometry of the gelatinolytic bands was performed on photographic negatives of the gels with a Bio-Rad Model 620 Video Densitometer (Bio-Rad, Richmond, VA).

Statistics

Each experiment was performed at least twice. Significance of differences between medians (ranges) was determined by the Mann-Whitney test and between means ± SEM by unpaired Student's *t* test.

RESULTS

In vivo evidence for bone matrix degradation by B16/F1 cells

To examine osteolysis in metastatic lesions, 7 mice were each intraarterially injected with 10^5 murine B16/F1 melanoma cells. Two weeks later, macroscopic and microscopic examination identified metastases in bones (100% of animals), ovaries (100%), adrenals (71%), lungs (57%), brain (43%) and kidneys (14%). The most frequently affected bones were femurs, tibias, mandible and maxilla in 100% mice, lumbar vertebrae (71%) and humerus (43%).

Histomorphometry of bone metastases showed 70% decrease in bone matrix, when compared with tumour-negative bones (9 ± 2% *versus* 28 ± 4%, respectively; $P<0.005$, Table 1). This was associated with replacement of the bone marrow by tumour cells ($P<0.0001$). Metastatic cells were frequently observed in

immediate contact with resorbed bone surfaces (Figure 1). In metastatic bones, 71 ± 8% of trabecular bone surfaces were covered by cancer cells (Table 1). This was associated with a 90% decrease in the proportion of osteoclasts, from 9 ± 3% in normal bones to 1 ± 1% in metastatic bones (P<0.05) and virtual elimination of osteoblasts (P<0.0001). Additionally, 96 ± 4% of all resorbed areas were covered by cancer cells, in contrast with 4 ± 4% located below osteoclasts (Table 1). To further assess the presence of osteoclasts in bone metastasis, TRAP activity was detected on plastic-embedded femurs recovered 5, 10 and 15 days post-injection of 10^5 B16/F1 cells. The proportion of TRAP-positive osteoclasts was reduced from 49 ± 3% in non-tumoral bones, to 7 ± 7% in metastatic bones at day 15 (P<0.01; Figure 2).

In vitro evidence for bone matrix degradation by B16/F1 cells

To investigate the mechanisms involved in direct osteolysis by tumour cells, we used *in vitro* models of matrix degradation. Since human osteosarcoma cell lines conserve the osteoblastic phenotype at various stages of differentiation (19), we used ^{3}H-proline-labelled extracellular matrices from human Saos-2 and U-2 OS cells as substrates for degradation. Exposure of these matrices to 10^5 B16/F1 cells or their CM increased ^{3}H release over control (Table 2). Cells induced higher matrix degradation than CM (P<0.05). When Saos-2-derived extracellular matrices were

mineralized in the presence of 10 mM β-glycerophosphate (14), we observed TPA-inducible degradation by B16/F1 cells and their CM (Table 2).

Radiolabelled rat fetal calvariae were used to determine if B16/F1 cells could degrade mineralized bone matrix. Consequently, ^{45}Ca-labelled calvariae were prepared and frozen/thawed to eliminate the possibility of interactions between tumour and bone cells. Devitalized calvariae were then exposed to 10^5 B16/F1 cells or their CM for 24 hours. B16/F1 cells and their CM significantly increased ^{45}Ca release (373 ± 10 cpm and 403 ± 8 cpm, respectively) from control release (329 ± 10 cpm; $P<0.01$ and $P<0.0001$, respectively). To determine if B16/F1 cells could form resorption pits on mineralized bones, we used cortical bone slices as substrates. As observed in Figures 3 and 4, pit formation was evident in bones exposed to B16/F1 cells for 3-10 days. These pits were focal depressions surrounded by a continuous rim and with a maximal diameter of 12-15 μm (area, 113 to 178 μm^2). Pits were not observed in bones incubated with medium alone (Figure 3, top panel).

In vitro evidence for a role of matrix metalloproteinases in matrix degradation by B16/F1 cells

Since activation of MMPs depends on the presence of divalent cations, the use of a chelator as enzymatic inhibitor denotes a role of these enzymes or other

metalloproteinases in functional assays (20). Degradation of ^{3}H-proline-labelled extracellular matrices by B16/F1-CM was inhibited by 1,10-phenanthroline. As observed in Table 3, addition of 1-50 mM 1,10-phenanthroline reduced matrix degradation up to 43% from control ($P<0.0001$).

Degradation of native type I collagen is known to be catalyzed by interstitial collagenase, collagenase-3 (21) and gelatinase A (9). Thus, we used tritiated native type I collagen as an *in vitro* substrate for tumour cells. B16/F1-CM increased type I collagen degradation 4-fold over background ($P<0.0001$, Figure 5). This degradation was further enhanced with B16/F1-CM from cells pretreated for 24 hours with 400 ng/ml TPA ($P<0.001$). Also, B16/F1 cells induced higher degradation than B16/F1-CM ($P<0.05$). TPA-pretreatment of B16/F1 cells with TPA enhanced their ability to degrade type I collagen.

Matrix metalloproteinase release by B16/F1 cells was documented by enzymography of B16/F1-CM. We observed gelatinolysis at M_r 90,000, 84,000 and 64,000 (Figure 6), and caseinolysis at M_r 54,000 (data not shown). The migration patterns of these bands correspond to those of gelatinases A and B, and interstitial collagenase or stromelysin (22). These bands disappeared after gel incubation with chelators of divalent cations such as EDTA (15 mM) or 1,10-phenanthroline (10 mM) for 48 hours at 37°C (data not shown). Since phorbol esters induce synthesis and release of MMPs in a variety of cell lines, and TPA increased matrix

degradation by B16/F1 cells, we exposed these cells to 20-400 ng/ml TPA for 24 hours and performed enzymography of their CM. As observed in Figure 6 and Table 4, induction of three gelatinolytic species of B16/F1-derived MMPs was observed, corresponding to the secreted and activated forms of gelatinase B and activated form of gelatinase A ($P<0.01$ for the M_r 90,000 band). A similar induction was observed with the M_r 52,000 caseinolytic band.

DISCUSSION

Bone metastases frequently exhibit osteolysis, a radiological indicator of their presence, and are associated with pain, fractures, hypercalcemia and mechanical alterations. Tumour cells have previously been shown to induce bone destruction systemically by secreting humoral resorptive factors or locally by releasing paracrine mediators of bone degradation (2,3). These factors activate osteoclasts by direct interaction with specific receptors or indirectly by stimulating osteoblasts that will release osteoclast activators (23). Thus, osteoclasts have been considered as major mediators of osteolysis associated with bone metastasis. However, it has been suggested that this might not be a universal mechanism, since experimental and human studies have shown that osteoclasts are only present at early stages of metastatic development (24-26) and will eventually be replaced by tumour cells. Consequently, tumour macrophages and metastatic cells

have been proposed as alternate cellular mediators during this "osteoclast-independent phase" of osteolysis (5,27). Specifically, macrophages (28) and human breast cancer and leukemic cells can directly destroy bone (6). In this study, we implicate metastatic melanoma cells and their matrix metalloproteinases as direct mediators of osteolysis.

An animal model of experimental bone metastasis by murine melanoma cells allowed us to perform histomorphometry of bone metastasis and revealed osteolysis at sites of bone metastasis. Osteolysis was not only locally associated with a striking reduction in the osteoclastic and osteoblastic populations, confirmed by a decrease in TRAP-positive osteoclasts, but also occurred in TRAP-negative locations where melanoma cells were in close association with bone surfaces. These findings demonstrated that local osteolysis occurred in this animal model and suggested direct involvement by murine melanoma cells. The presence of tumour cells close to resorptive surfaces does not necessarily mean that these cells are responsible for resorption. However, histomorphometric evidence of bone degradation together with virtual absence of osteoclasts and a local predominance of tumor cells replacing the bone marrow, clearly supports a role of metastatic cells as direct mediators of osteolysis at this stage of metastatic development.

Since osteosarcomas have an osteoblastic origin and typically produce osteoid rich in type I collagen, we used matrices derived from osteosarcoma cell

lines as *in vitro* substrates for metastatic melanoma cells. Our data showed that, under the *in vitro* conditions used in our experiments, B16/F1 cells and secreted factors in their conditioned medium degraded such osteoid-like matrices. The ability of B16/F1 cells to degrade mineralized matrices was tested on three different bone preparations, including osteosarcoma-derived matrices exposed to β-glycerophosphate, rat fetal calvariae and bovine bone slices. These experiments indicated that murine melanoma cells degrade mineralized bone matrices. Rat fetal calvariae, labelled with ^{3}H-proline or ^{45}Ca, have been previously used for studies of osteoclastic degradation (17,29). In fact, degradation by tumour cells was previously demonstrated *in vitro* by Eilon and Mundy (6), who showed that human cell lines induced release of radioactive calcium from rat fetal calvariae. Our experiments confirm their findings with murine melanoma cells using osteoid-like and bone substrates as the targets.

Our evidence for formation of resorption pit assays by melanoma cells contrasts with previous studies in which tumor cells *per se* did not appear to directly degrade bone. In particular, Boyde *et al.* (30) based this conclusion on unspecified ultrastructural features of resorption pits from a limited number of human spongy bone samples, in which tumor and bone cells were removed to identify the resorptive areas. Quinn *et al.* (28) addressed this question *in vitro* and showed that rat osteosarcoma cells were able to produce resorption pits in the presence of tumor-infiltrating macrophages and active vitamin D. Although they did not observe

pit formation by carcinoma cell lines, details as to the culture conditions do not unequivocally rule out this possibility.

Interstitial collagenases belong to the family of MMPs known to degrade type I collagen. In our study, a role for MMPs in tumour-induced osteolysis was demonstrated *in vitro* by: (a) abrogation of tumour-induced matrix degradation by a chelator of divalent cations, (b) degradation by melanoma cells of purified type I collagen as a specific substrate for MMPs, and (c) enhancement of degradation by the phorbol ester TPA. Enzymography confirmed that this was associated with increased release by melanoma cells of active MMPs. Although the migration patterns observed in enzymography correspond to those of human gelatinases, interstitial collagenase and/or stromelysin, their identification will rely ultimately on the use of specific anti-mouse antibodies. Since TPA is responsible for the induction of many cellular pathways, the degradative induction observed may not necessarily be all mediated by MMPs, but rather by a combination of multiple mechanisms including these enzymes. That other degradative mechanisms are involved is also supported by the observation that *in vitro* inhibition of MMPs by a cation chelator only achieved partial inhibition of matrix degradation. Additional mechanisms also responsible for degradation may include other enzymatic systems capable of degrading matrix components such as the serine, aspartic or cysteine proteinases. Also, since chelating agents inhibit other cation-dependent metalloproteinases, the use of purified TIMPs would be ideal to determine the role

of MMPs in this degradative phenomena. Thus, we suggest that tumor-derived MMPs are mediators, at least in part, of direct osteolysis by murine melanoma cells.

The mechanisms by which cancer cells induce demineralization of bone matrix are not known. Osteoclasts degrade bone by a combined action of membrane proton-channels and proteinases, resulting in extracellular acidification, matrix demineralization and degradation (31). The presence of similar mechanisms on cancer cells has not been reported. We speculate that cancer cells may acidify their immediate microenvironment by other yet undefined mechanisms, in a less efficient way than osteoclasts. Additionally, although cancer cells express type I collagen-binding integrins, it is unlikely that they will produce focalized degradation analogous to the $\alpha_V\beta_3$-mediated osteoclastic osteolysis, since osteoclasts have a complex machinery specialized in this task. Therefore, we propose that bone-metastasizing cells, which secrete MMPs and induce matrix demineralization can cause direct osteolysis, although it may be of differing efficiencies and morphology than that due to osteoclastic action.

Three cellular mediators of osteolysis have been proposed, namely, osteoclasts (32), tumor macrophages (28) and cancer cells (6,27). The present study demonstrates a role of cancer cells and their MMPs in directly causing bone matrix degradation. Classic observations in bone metastasis by VX2 carcinoma cells led Galasko to propose a "two-phase hypothesis" of osteolysis, with an

"osteoclast-dependent" phase being followed by an "osteoclast-independent" phase, in which tumour cells and/or tumour macrophages directly participate in the destructive process (25,33,34). This "dual nature" of osteolysis is accepted but considered applicable only on a tumour-specific basis. Our data suggest and support the "osteoclast-independent" phase. That MMPs are involved in tumor osteolysis is important since novel therapeutic approaches could be derived. Future characterization of the MMPs responsible for bone degradation may involve the use of blocking antibodies or specific enzyme inhibitors. This study may help to explain why antiosteoclastic agents do not completely prevent or abrogate metastasis-associated local osteolysis (35). Since tumor cells cause direct bone degradation, bisphosphonate treatments which inhibit osteoclastic activity, should be complemented by inhibitors of matrix metalloproteinases.

In conclusion, this study demonstrates direct bone matrix degradation by melanoma cells and suggests this effect may be mediated by MMPs. These observations provide evidence for the concept that osteoclasts, tumour-associated macrophages *and* cancer cells directly participate in local bone matrix degradation associated with bone metastasis.

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TABLE 1. HISTOMORPHOMETRIC EVALUATION OF B16/F1 BONE METASTASIS

	Tumor Positive[a] n=7	Tumor Negative n=7	*t* Test
	Mean ± SEM	Mean ± SEM	*P* Value
Area[b]			
Bone	9 ± 2%	28 ± 4%	0.0026
Bone Marrow	1 ± 1%	72 ± 4%	<0.0001
Tumor	90 ± 2%	---	---
Cells on Trabecular Surfaces[c]			
Osteoblasts	0 ± 0%	30 ± 6%	<0.0001
Osteoclasts	1 ± 1%	9 ± 3%	0.0480
Tumor Cells	71 ± 8%	---	---
Resting	28 ± 7%	61 ± 7%	0.0060
Resorption Pits[d]			
Osteoclasts	4 ± 4%	100 ± 0%	<0.0001
Tumor Cells	96 ± 4%	---	---

[a] Seven C57BL/6 mice were injected intraarterially with 10^5 B16/F1 melanoma cells. Two weeks later, the animals were killed and the right distal femoral epiphysis decalcified and histologically processed. Seven sex- and age-matched animals without bone tumors were used as controls.

[b] Values represent areas of bone, bone marrow and tumor, expressed as percentages of total area examined.

[c] Values represent proportions of cell subpopulations on the trabecular surfaces, expressed as percentages of the total number of surfaces counted. The surfaces counted were those intersected by lines in the Merz graticule.

[d] Values represent proportions of bone resorption pits overlaid by cancer cells or osteoclasts, expressed as percentages of total number of pits counted. Resorption pits counted were those intersected by lines in the Merz graticule.

TABLE 2. DEGRADATION OF Saos-2 AND U-2 OS EXTRACELLULAR MATRICES BY B16/F1 MELANOMA CELLS[a]

Conditions	Saos-2 Matrix	U-2 OS Matrix	Saos-2 Matrix *plus* β-GPO$_4$[e]
Unconditioned Medium	99 ± 6	251 ± 24	780 ± 41
B16/F1-CM	123 ± 9[b]	313 ± 16	1199 ± 63
B16/F1 Cells	115 ± 2[b]	614 ± 109[c]	1511 ± 33
TPA-Treated Cells	144 ± 15[b]	859 ± 110[d]	1998 ± 88
CM from TPA-Treated Cells	----	----	1504 ± 39

[a] Extracellular matrices from human Saos-2 and U-2 OS osteosarcoma cells were metabolically labelled with ^{3}H-proline. Cells were then lysed with 20 mM NH_4OH. Matrices were incubated with either 200 μl B16/F1-CM, 10^5 B16/F1 cells or 10^5 B16/F1 cells treated with 400 ng/ml TPA. Twenty-four hours later, release of ^{3}H was determined from medium aliquots. Each value represents the mean ± SEM of 8 independent replicates and is expressed as disintegrations per minute.

[b] $P<0.05$ for test conditions *vs.* unconditioned medium.

[c] $P<0.05$ for B16/F1 cells *vs.* unconditioned and CM.

[d] $P<0.0001$ for TPA-treated cells *vs.* unconditioned medium.

[e] Partial mineralization of Saos-2 matrices was obtained by incubating Saos-2 cells in the presence of 10 mM β-glycerophosphate (**β-GPO$_4$**) for 2 weeks (14). Crystal formation was evident after 1 week of incubation. $P<0.0001$ for B16/F1-CM and cells *vs.* unconditioned medium. $P<0.005$ for B16/F1 cells *vs.* CM. $P<0.0001$ for B16/F1 cells treated with 400 ng/ml TPA *vs.* untreated cells. $P<0.05$ for CM from B16/F1 cells treated with 400 ng/ml TPA *vs.* CM from untreated cells.

TABLE 3. EFFECTS OF 1,10-PHENANTHROLINE ON THE DEGRADATION OF U-2 OS MATRIX BY CONDITIONED MEDIUM FROM B16/F1 MELANOMA CELLS

Conditions[a]	^{3}H-Proline Release	Percent Inhibition	*P* Value
Serum-Free MEM	384 ± 14	----	----
B16/F1-CM	922 ± 31	----	----
B16/F1-CM + 1 mM PNAT	837 ± 58	9%	NS
B16/F1-CM + 10 mM PNAT	751 ± 34	19%	0.0029
B16/F1-CM + 50 mM PNAT	530 ± 19	43%	<0.0001

[a] Extracellular matrices from human U-2 OS osteosarcoma cells were metabolically labelled with ^{3}H-proline. Cells were then lysed with 20 mM NH_4OH. Matrices were incubated with 200 μl B16/F1-CM in the presence of increasing concentrations of 1,10-phenanthroline, for 24 hours at 37°C. Release of ^{3}H was then determined from medium aliquots. Each value represents the mean of 8 independent replicates and is expressed as disintegrations per minute.

TABLE 4. INDUCTION OF MATRIX METALLOPROTEINASE RELEASE BY TREATING B16/F1 CELLS WITH TPA

Molecular Weights[a]	TPA 20 ng/ml[b]	TPA 100 ng/ml	TPA 400 ng/ml
90,000 Da	7% (0-14)	16% (11-19)[c]	19% (16-24)[c]
84,000 Da	5% (2-7)	10% (7-12)	9% (7-11)
64,000 Da	1% (0-6)	5% (0-12)	6% (0-14)

[a] Cancer cell monolayers were incubated with serum-free medium in the presence of 20-400 ng/ml TPA for 24 hours. Samples of B16/F1-CM were then collected. Protein-standardized samples were resolved by electrophoresis in a 10% SDS-PAGE containing 0.3% gelatin. Gels were partially digested with 2.5% Triton X-100 for 30 minutes. Enzyme degradation of the substrate was allowed to occur in a buffer containing 0.1 M Tris-HCl pH 8.0, 5 mM $CaCl_2$ and 0.04% NaN_3, for 48 hours at 37°C. Gels were then stained with 2.5% Coomassie Blue for 30 minutes, and destained with methanol/acetic acid/water (40:10:50). Densitometry of the gelatinolytic bands was performed on negative photographs of the gels.

[b] Results represent percent increases over control (TPA 0 ng/ml), and are expressed as median (range) from 6 different experiments.

[c] Significantly different from untreated cells by Mann-Whitney test. $P<0.05$ for TPA 100 ng/ml and $P<0.01$ for TPA 400 ng/ml.

FIGURE LEGENDS

FIG. 1. Experimental femoral bone metastasis by B16/F1 cells 14 days after intraarterial injection of 10^5 cells. Melanoma cells are located directly over a partially-resorbed bone surface (arrow). Osteoclasts, osteoblasts or bone marrow cells are noticeably absent. x250, hematoxylin and eosin.

FIG. 2. Proportion of bone and tumor cells in bone metastasis. Detection of TRAP activity was performed in plastic-embedded femurs at days 0, 5, 10 and 15 after intraarterial injections of 10^5 murine B16/F1 melanoma cells. The number of osteoclasts was reduced in metastatic bones at days 10 and 15 ($P<0.01$).

FIG. 3. Resorption pits on bone surfaces exposed to B16/F1 melanoma cells. Scanning electron microscopy of the surface of longitudinal bovine cortical bone slices displayed depressions demarcated by a continuous rim and with a diameter of 12-15 μm (**bottom panel**). Pits were not observed in bones incubated with medium alone (**top panel**) Bar = 10 μm.

FIG. 4. Presence of bone resorption pits on bovine bone slices exposed to B16/F1 melanoma cells. The percent area of bone slices with resorption pits was determined by light microscopy according to Ritchie *et al.* (17). The presence of tumor cells was associated with a time-related increase in the areas of bone slices displaying resorption pits. Values represent the mean ± SEM of the proportion of resorption pits present in three bones *per* time period.

FIG. 5. Degradation of type I collagen by B16/F1 cells. Conditioned medium or B16/F1 cells placed onto ^{3}H-labelled type I collagen induced radiolabel release ($P<0.0001$ for test conditions *vs.* unconditioned medium). Challenge of the tumor cells with 400 ng/ml TPA further increased collagenolysis ($P<0.001$ for CM from TPA-treated cells *vs.* B16/F1-CM). **CLS**, cells, **CM**, conditioned medium, **MEM**, serum-free Minimum Essential Medium.

FIG. 6. Gelatin enzymography of B16/F1-derived conditioned medium. Unstimulated B16/F1 cells displayed gelatinolytic activity at M_r 90,000, 84,000 and 64,000. These activities were enhanced by exposure to increasing concentrations (0-400 ng/ml) of the phorbol ester TPA.

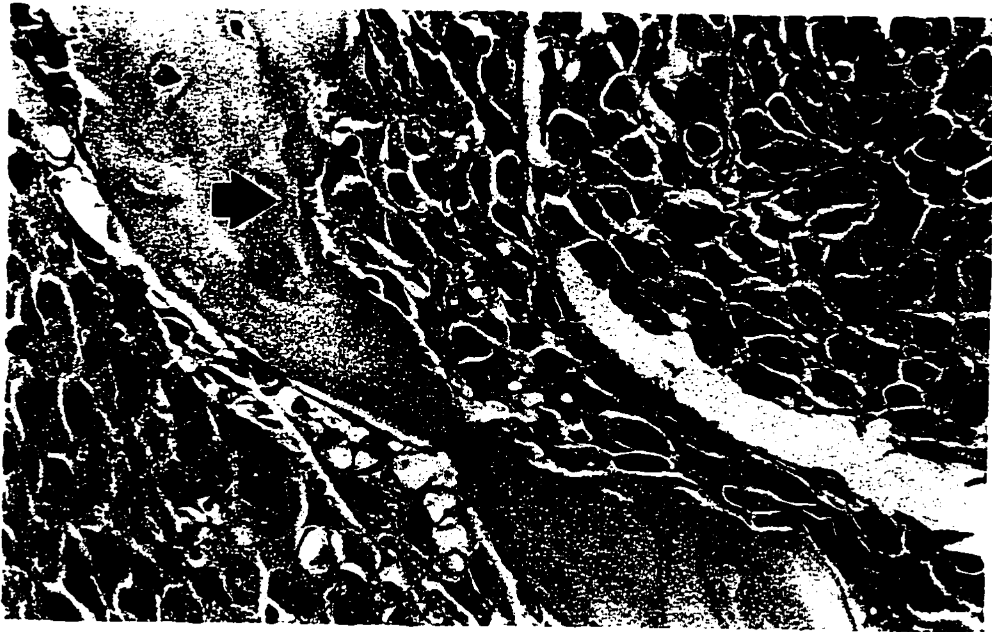


Figure 1

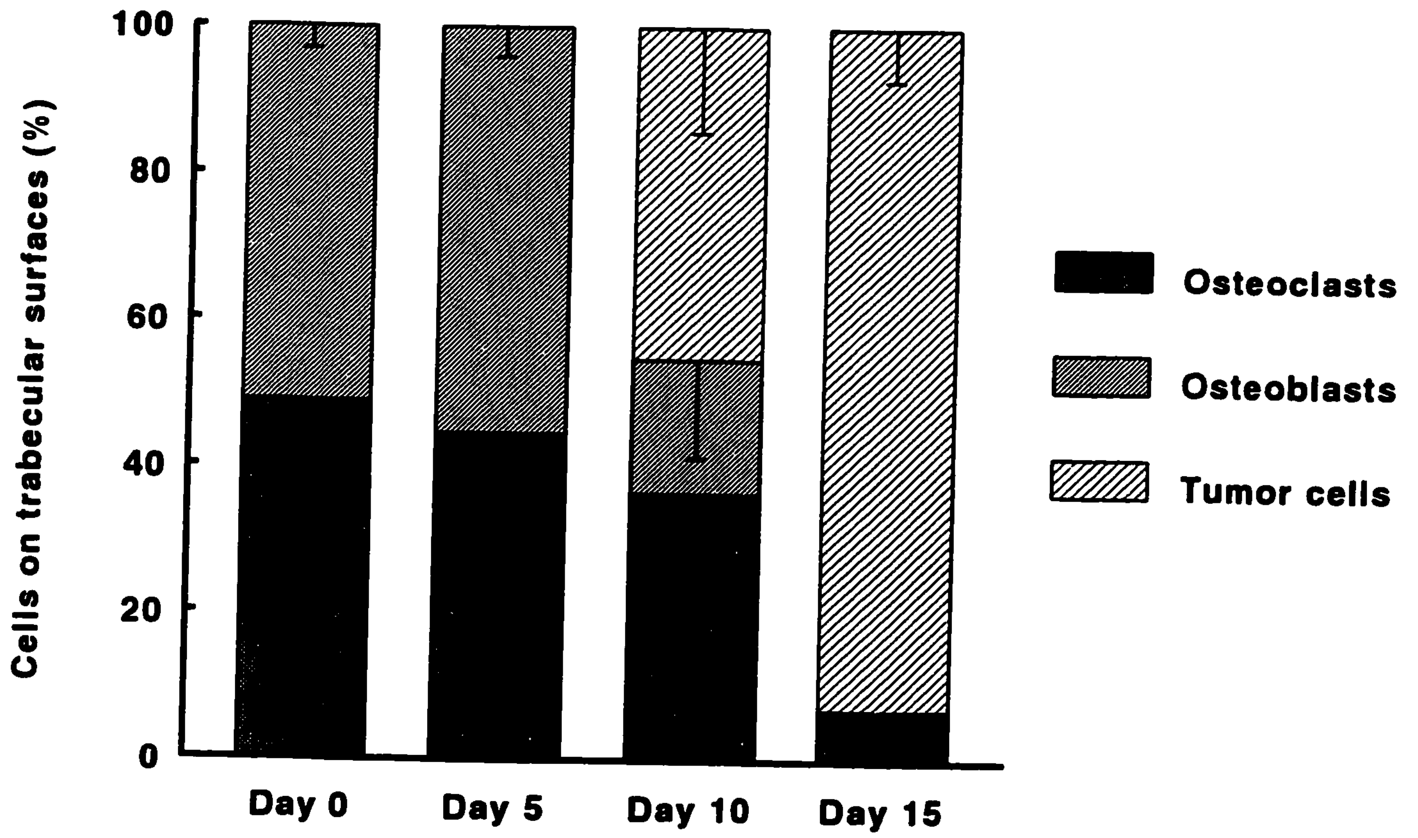


Figure 2

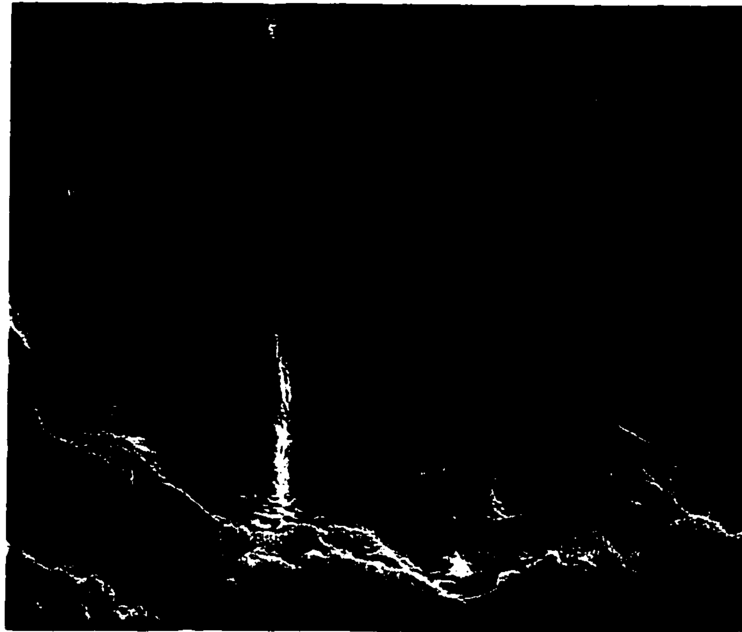
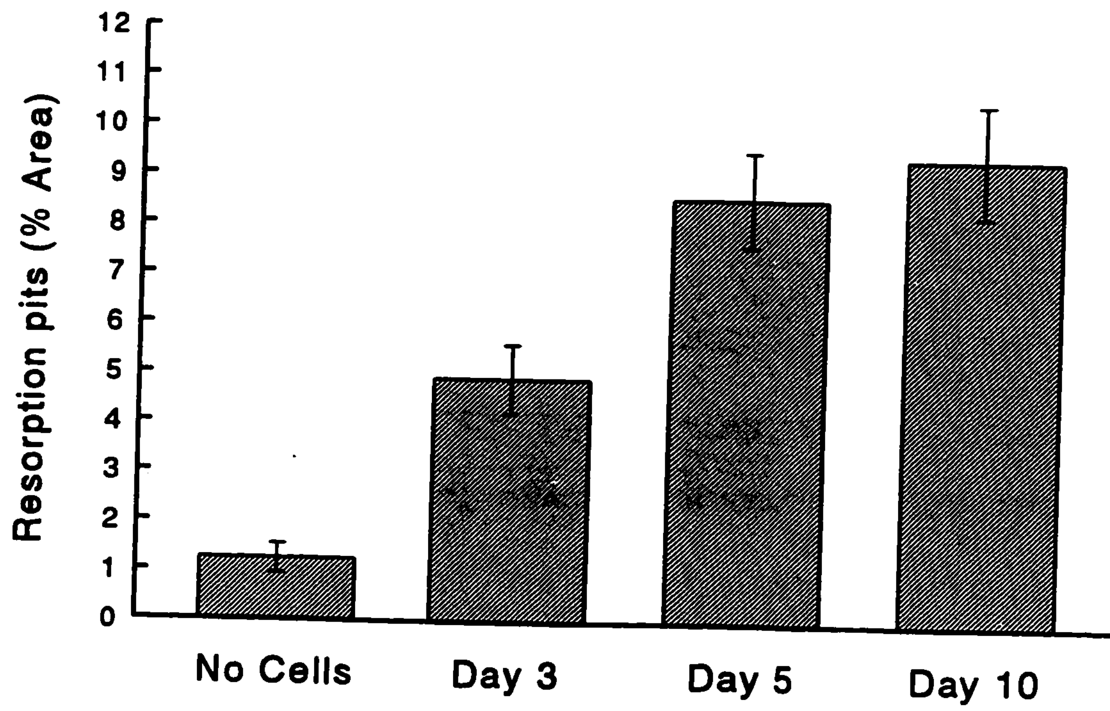


Figure 3



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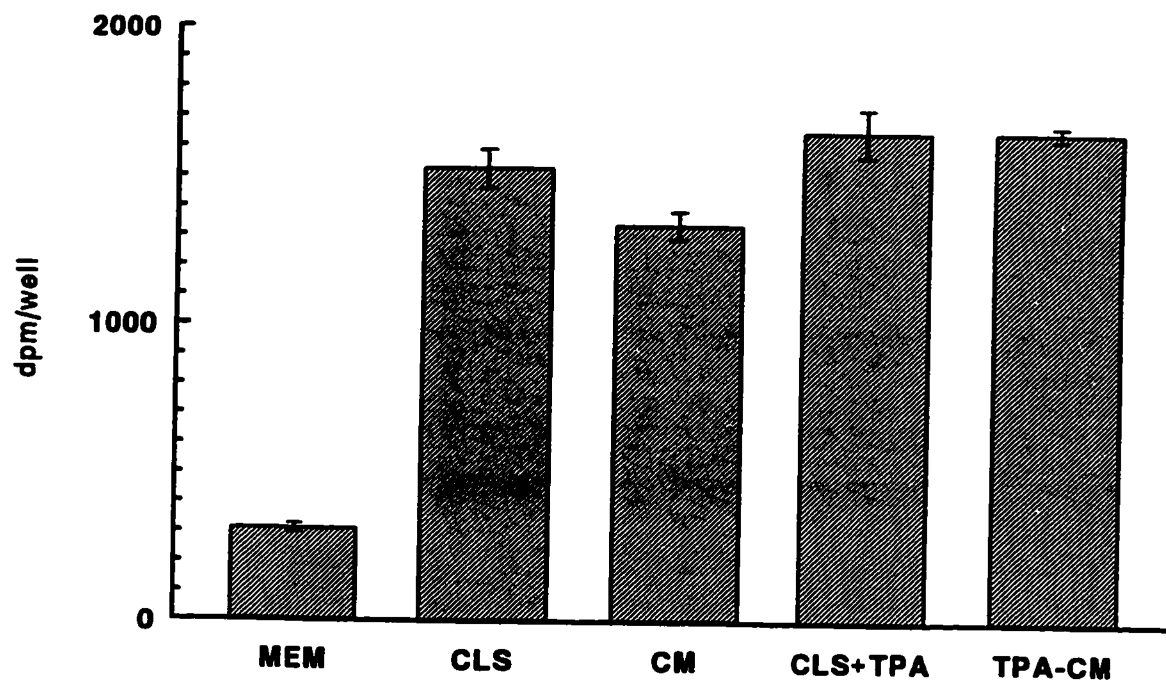

Figure 5

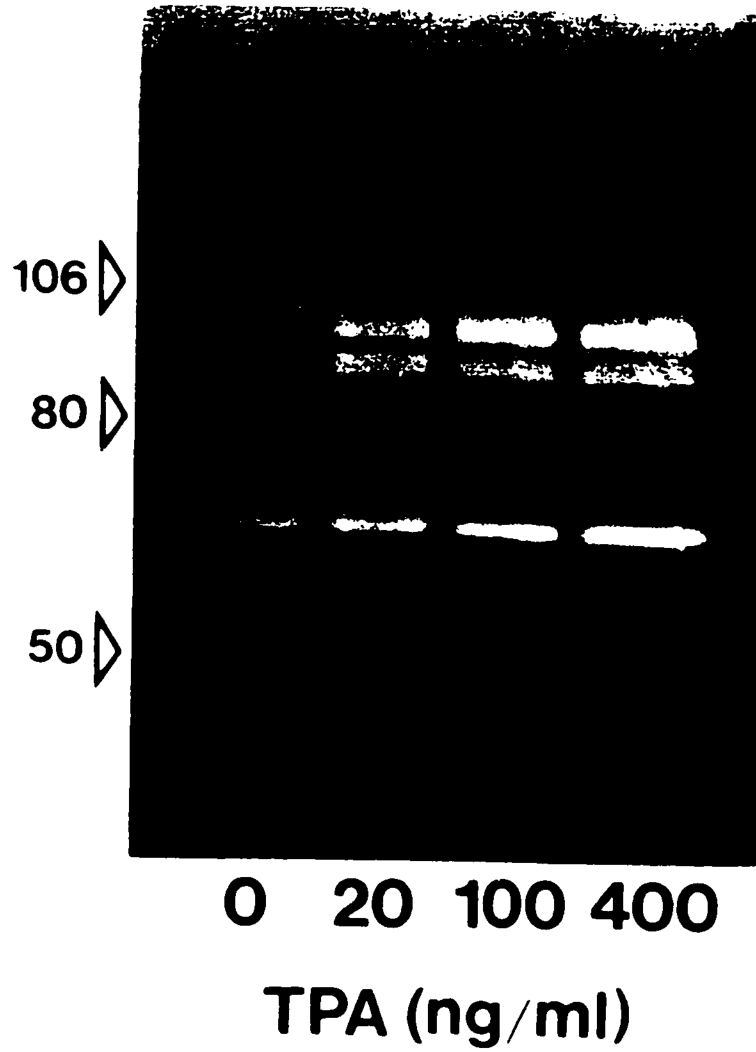

Figure 6

3. Additional Results and Discussion

Analysis of interactions between tumor cells and bone matrix require a combined approach that uses *in vivo* and *in vitro* techniques. For the study of metastasis, the use of animal models has the advantage of demonstrating the natural behaviour of tumor cell lines in different organ sites. Murine melanoma B16/F1, when injected into the arterial system, developed metastasis in different organs including bones in 100% of animals successfully injected. The histological appearance and histomorphometric analysis of the skeletal metastases, suggested that these cells could induce osteolysis in association with a significant proportional reduction in osteoclasts or other bone cells. This observation was confirmed by *in vitro* experiments that used different types of bone-related extracellular matrices as degradation targets.

Difficulties with the interpretation of these results, common to many *in vitro* approaches, stem from the use of matrices that are either non-mineralized or are mineralized but not viable. Because of technical limitations, It is not yet possible to consistently use viable bone matrix in culture for adequate periods of time (Mundy et al., 1991). Furthermore, each one of the techniques used has some kind of limitation. For instance, the use of ^{3}H-labelled osteosarcoma-derived matrices, although similar to osteoid and easily quantifiable, is not ideal since these matrices cannot reproducibly and within a reasonable time frame be mineralized. Attempts

at inducing mineralization by exposure to β-glycerophosphate proved to be inconsistent. Although the use of ^{3}H-proline as label is adequate, measurement of ^{3}H release may not be considered ideal, since it may include "free" or not metabolically incorporated radioisotope. Some authors have instead used measurement of ^{3}H-hydroxyproline release, a more demanding and not practical endpoint, since it requires chromatographic identification of the hydroxylated amino acids. The *in vitro* approaches used here are, thus, complementary: the use of different degradation substrates allowed us to study the proteolytic behaviour of bone-metastasizing cells on devitalized bone and bone-related matrices.

Mineralized bone labelled with radioactive calcium (^{45}Ca) has been traditionally used as substrate for bone resorption (Fukushima et al., 1983; Mundy et al., 1991; Chambers et al., 1984; Blair et al., 1986; Raisz, 1965). Experiments performed by Mundy *et al.*, and our studies, show that when these bones are devitalized and incubated with cancer cell lines *in vitro*, there is increased release of ^{45}Ca into the medium (Mundy et al., 1977; Koeffler et al., 1978; Eilon et al., 1983; Eilon et al., 1978; Mundy et al., 1978). This effect may be caused by cancer cells directly inducing calcium removal or indirectly causing bone matrix degradation, resulting in secondary mineral release. The difficulty with these two interpretations is that, based on current concepts in tumor biology, it is not possible to conceive a mechanism(s) by which cancer cells might directly induce demineralization, degrade mineralized bone matrix, or remove released calcium. Whatever the

mechanism(s), it is accepted that cancer cells will not be as efficient as osteoclasts, cells specialized in matrix demineralization and subsequent degradation (Baron, 1989).

Tissue inhibitors of matrix metalloproteinases (TIMPs) are important in limiting tumor invasion and metastasis (DeClerck et al., 1994). Taking advantage of the availability of murine melanoma B16/F10 cells transfected with and overexpressing TIMP-1, with Dr. Rama Khokha, London Regional Cancer Centre, we decided to test if overexpression of TIMP-1 in these melanoma cells would affect their metastatic behaviour in the bone. For this purpose, we used these TIMP-1 transfectants to produce experimental bone metastasis. This was followed by histomorphometry of skeletal lesions. In brief, four cell clones were used: (1) B16/F10 wild type cells (n=6), (2) B16/F10 clone 1-2 (n=13), used as negative control, since it was transfected with plasmid, (3) B16/F10 clone 2-8 (n=13), transfected with and overexpressing TIMP-1, and (4) B16/F10 clone 6-5 (n=14), similar to clone 2-8, but shown to constitutively overexpress TIMP-1 in a higher degree that clone 2-8. The tumorigenic and metastatic abilities of TIMP-1 transfectants were previously tested *in vivo*: these cells displayed fewer lung metastases than wild type or plasmid-transfected cells (Khokha et al., 1992; Khokha, 1994). In this project, following left ventricular injection of 10^5 cells/animal, tumor involvement was similar to that observed previously with non-transfected clones. After death, the total number of metastatic tumors in bone *per* animal was:

6 ± 1% for wild type, 14 ± 2% for clone 1-2, 8 ± 1% for clone 2-8 and 12 ± 3% for clone 6-5 (Table 1). Thus, measuring this parameter, only one of the TIMP-1 transfected clones (2-8) showed a decreased number of bone metastasis than the plasmid-transfected clone (negative control, $P<0.02$). This difference was also maintained when visceral metastases were included: the *total* number of metastasis *per* animal was 16 ± 1% for 1-2 *versus* 10 ± 2% for 2-8 cells, a TIMP-1 transfectant ($P<0.05$). Histomorphometry of femoral metastases, however, revealed that this difference between the negative control 1-2 and the TIMP-1 transfectant 2-8 were not reflected on their osteolytic capacity (Table 1). In fact, no significant differences in osteolysis were observed with the TIMP-1 transfectants. These findings suggested, then, that TIMP-1 expression does not have any influence in metastasis-associated osteolysis, or if it does, it is not a single determinant of the osteolytic ability of cancer cells.

In summary, the in vivo and in vitro studies described in the manuscript and additional results demonstrated that murine B16/F1 melanoma cells are capable of inducing bone resorption and degradation of bone-related matrices, and suggest that tumor-derived matrix metalloproteinases may be mediators of this process.

Table 1. Histomorphometry of bone metastasis by TIMP-1 transfectants of murine B16/F10 melanoma cells

	Plasmid	n	Number of Metastases in bones	Bone Volume	Tumor volume	Bone marrow volume	Osteoblasts	Osteoclasts	Tumor cells	Resting cells
No tumor	---	7	6	9±2%	0±0%	91±2*	18±5%*	3±1%*	0±0%	79±5%*
B16/F10	---	3	14	4±0%	88±6%	8±6%	2±1%	0±0%	36±6%	62±7%
Clone 1-2	Control	10	8	7±1%	71±11%	22±10%	3±1%	1±1%	35±6%	61±5%
Clone 2-8	TIMP-1	9	12	6±1%	68±13%	26±12%	3±3%	1±1%	45±9%	51±7%
Clone 6-5	TIMP-1 (higher)	5		7±1%	77±14%	16±15%	0±0%	0±0%	45±10%	54±9%
P value				NS	NS	*<0.01	*<0.05	*<0.05	NS	*<0.05

D. OSTEOLYSIS BY HUMAN BONE-METASTASIZING CELLS

1. Introduction

The use of non-human tumor cells to study the pathogenesis of bone metastasis has, historically, provided the advantages of easy accessibility to cell lines, a wide diversity of cell lineages and types, and use of immunocompetent animals as hosts. In fact, most of the experimental evidence and current theories on pathogenetic mechanisms of bone metastasis are based on the use of mouse, rat and rabbit models. This has hindered swift extrapolation of these findings to clinical situations (Olden, 1990).

In contrast, the experimental study of human bone metastasis is, in principle, handicapped. Although, *in vivo* use of human cell lines has been possible with immunocompromised mice or rats, there is a concern, that these models do not reflect clinical situations. Moreover, immunodeficient animals constitute, by definition, abnormal hosts. Thus, the study of interactions between human metastatic cells and their surrounding host microenvironment in these animals, must be interpreted cautiously. For instance, regarding bone metabolism, the bones of athymic mice display altered morphometric features when compared with non-immunocompromised counterparts. More specifically, 12-week-old athymic mice, in comparison with euthymic mice, had smaller vertebral area and tibia length,

decreased osteoclast number, reduced double labeled perimeter (indicating reduced bone formation), reduced alkaline phosphatase levels and decreased femoral ash weight (McCauley et al., 1989).

Human cell lines obtained from bone metastasis have been used to address through *in vitro* experiments the nature of interactions between these cells and their surrounding matrix. Three such cell lines have been available during the last two decades. They include prostate PC-3 adenocarcinoma cells, by far the most studied of the three, SK-N-SH neuroblastoma cells, the first to be isolated in 1973, and Hs696 adenocarcinoma cells, of unknown primary origin. These three cell lines were used in this project to address questions similar to those addressed with the murine B16/F1 melanoma cells. Specifically, the degradative capacity of these cells was assessed using bone and bone-related matrices, as well as their production of matrix metalloproteinases.

Human Skeletal Metastatic Cell Lines Directly Degrade Bone Matrix Using Matrix Metalloproteinases

Otto H. Sanchez-Sweatman, Gurmit Singh, Rama Khokha, Achim Krüger, Helga Duivenvoorden and F. William Orr

Department of Pathology and Oncology Research Group, McMaster University, Hamilton, Ontario [O.H.S.-S., H.D. and G.S.], Ontario Cancer Institute, Toronto, Ontario [R.K. and A.K.], and Department of Pathology, University of Manitoba, Winnipeg, Manitoba [F.W.O.], Canada

Submitted to: Cancer Research

Name and address for correspondence:

Dr. Gurmit Singh
Research, Hamilton Regional Cancer Centre
699 Concession Street
Hamilton, Ontario
Canada L8V 5C2

Tel: (905) 387-9711 Ext. 7007
FAX: (905) 575-6330

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Key words:

Metastasis, Bone, Matrix Metalloproteinases, Osteolysis.

ABSTRACT

Bone metastases are often associated with osteolysis and subsequent pathological fractures. We have previously shown that metastatic murine melanoma cells directly degrade bone matrices and identified tumor-derived matrix metalloproteinases as mediators. Here, we used three human cell lines established from bone metastatic sites: PC-3 prostate adenocarcinoma, SK-N-SH neuroblastoma and Hs696 adenocarcinoma. We show that PC-3 and SK-N-SH cells and their conditioned medium degraded radiolabelled extracellular matrices from human Saos-2 and U-2 OS osteoblast-like cells. Degradation of mineralized bone was evidenced by PC-3 cells and their conditioned medium inducing ^{45}Ca-release from rat fetal calvariae and forming resorption pits on bone slices, an effect increased by TGF-β1. A role for matrix metalloproteinases in degradation was shown by: (a) stimulation by the phorbol ester TPA of matrix degradation by PC-3 and SK-N-SH cells, of their release of M_r 92,000, 72,000 and 52,000 gelatinolytic activity, and of their MMP-1 mRNA levels; (b) abrogation of matrix degradation by 1,10-phenanthroline, a metalloproteinase inhibitor; and (c) degradation of purified type I collagen by PC-3 cells and their conditioned medium (increased 4-6 fold over control). Additionally, the release of matrix metalloproteinases by Hs696 cells was enhanced after a 24-hour incubation with 20 ng/ml purified human TGF-β1, a growth factor prevalent in the bone. We demonstrate that human cancer cells can directly degrade bone-related matrices and that matrix metalloproteinases have a role in this process.

INTRODUCTION

Bone metastases are frequently associated with osteolysis (1). A number of different cells have been proposed as direct mediators of bone matrix degradation including osteoclasts, osteoblasts, tumor-associated macrophages and cancer cells (2-5). However, the possibility that metastatic cancer cells might directly degrade osteoid and mineralized bone has not been widely studied. The direct involvement of cancer cells has been suggested on the basis of morphological observations after intramedullary injection of Brown-Pearce carcinoma cells in rabbit bones, showing local tumor-induced osteolysis in the absence of osteoblasts or osteoclasts (6). Subsequently, Mundy *et al.* demonstrated that human breast cancer cells (4) and malignant histiocytes (7) or their conditioned medium could directly induce release of radiolabelled calcium from devitalized rat fetal calvariae *in vitro* (8,9). Thus, these experiments provided evidence that in the absence of viable bone cells, cancer cells could degrade mineralized bone.

Matrix metalloproteinases are implicated in physiological bone degradation. While osteoclasts, specialized in bone degradation, produce matrix metalloproteinases such as interstitial collagenases and MMP-9 (10), osteoblasts, thought to assist in osteoid removal, synthesize and release collagenases and their inhibitors (11). It is reasonable then to assume that, since tumor cells produce

matrix metalloproteinases, a phenomenon essential for tumor invasion (12), they might use a similar mechanism to directly degrade bone.

This study demonstrates that human cancer cell lines established from bone metastases are able to directly degrade bone and bone-related matrices and provides data to support the hypothesis that tumor-derived matrix metalloproteinases are mediators of bone degradation.

MATERIALS AND METHODS

Tumor cell lines. The three human tumor cell lines used for these studies have been established from metastatic bone tumors and were obtained from the American Type Culture Collection (Rockville, MD). These were PC-3 prostate adenocarcinoma (13), SK-N-SH neuroblastoma (14) and Hs696 adenocarcinoma of unknown primary origin (15,16).

Preparation and use of osteosarcoma-derived extracellular matrices in degradation assays. Extracellular matrices derived from human Saos-2 and U-2 OS osteosarcoma cells (American Type Culture Collection, Rockville, MD) (17,18), were metabolically radiolabelled and exposed to different cancer cell lines or their conditioned medium. These matrices have been previously shown to contain

collagen (19). Radiolabelled extracellular matrices were prepared by culturing these cells in Minimum Essential Medium supplemented with 10% fetal bovine serum and antibiotics, with 5 μCi/ml ^{3}H-proline (NEN, Boston, MA) in 96-well plates for 5 days, at which time cell monolayers were confluent. Cells were then lysed with 20 mM NH_4OH at 37°C for 20 minutes, whereby the radiolabelled matrix remained at the bottom of the wells (20). After 3 rinses with phosphate-buffered saline, matrices were exposed to 10^5 tumor cells *per* well or 200 μl fresh conditioned medium *per* well. The release of ^{3}H into the medium was determined 24 hours later by collecting 50 μl aliquots into 3 ml scintillation fluid (Ready Safe™, Beckman, Fullerton, CA). Each experimental value was determined from 8 experimental replicates, establishing the mean ± standard error of the mean (SEM). These experiments were performed at least twice.

Preparation and use of rat fetal calvariae in degradation assays. To determine the degradation of mineralized bone, ^{45}Ca-labelled calvariae were prepared according to previously published protocols by Magro *et al.* (21). In brief, pregnant Sprague-Dawley rats were injected subcutaneously with 10 µCi of ^{45}Ca on day 15-16 of gestation. On day 20, fetal calvariae were dissected, rinsed and frozen to kill the endogenous bone cells. After a minimum of 24 hours, calvariae were thawed, rinsed and exposed to 10^5 tumor cells *per* well or 1 ml fresh tumor-derived conditioned medium *per* well for up to 96 hours. Release of ^{45}Ca into the medium was then determined. Each experimental value was determined from 4

experimental replicates, establishing the mean ± SEM. These experiments were performed twice.

Resorption pit assay. Longitudinal bovine cortical bone slices from the femoral midshaft were prepared according to Chambers *et al.* (22). Slices (5x5x2 mm) were cut with an Isomet low-speed diamond-edged saw (Buehler Instruments, Evanston, IL), cleaned by ultrasonication for 30 minutes, washed in ethanol, dried, and stored. After the slices were presoaked with serum-free medium, 10^5 PC-3 cells were placed onto the slices and incubated for 60 min at 37°C to allow cell attachment. Non-adherent cells were removed by rinsing with serum-free medium. Incubation in 5 ml MEM with 10% FBS and antibiotics at 37°C for 3-7 days, was followed by removal of cancer cells with 0.1% Triton X-100 in dH_2O for 6 hours, and fixation of the slices with 10% formaldehyde or 2.5% glutaraldehyde in PBS. Slices were stained with 1% toluidine blue for light microscopy according to Ritchie *et al.* (23). The area covered by resorption pits was determined with a Merz graticule (24) in ten different microscopic fields *per* sample and expressed as percentage of total bone surface area in these fields.

Enzymography of tumor-derived conditioned medium. Cancer cell monolayers were cultured to near-confluency in serum-supplemented medium. Rinsing with PBS was followed by incubation with serum-free MEM and antibiotics with 0-400 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 24 hours.

Samples of tumor cell-derived conditioned medium were collected and centrifuged to remove cell debris. Protein concentration in each conditioned medium was determined by Bradford's protein assay (Bio-Rad, Richmond, CA). Protein-standardized samples were resolved at 4°C in 10% SDS-PAGE containing 1 mg/ml gelatin or casein (Sigma, St. Louis, MO) (25). For this purpose, gelatin was solubilized in water by warming to 60°C while stirring. Similarly, casein was solubilized in 200 mM NaOH and neutralized by adding 1.5 M Tris-HCl pH 8.8. After gel electrophoresis, incubation with 2.5% Triton X-100 for 30 minutes was followed by enzymatic degradation of the substrate in a buffer containing 0.1 M Tris-HCl (pH 8.0), 5 mM $CaCl_2$ and 0.04% NaN_3, for 48 hours at 37°C. Gels were stained with 2.5% Coomassie Blue for 30 minutes, and destained with methanol/acetic acid/water (40:10:50).

Protein immunodetection. MMP-2 proteins were visualized using western blot analysis. After conditioned media from Hs696 cells were resolved in 10% SDS-PAGE, proteins were transferred onto a Hybond-C nitrocellulose membrane (Amersham, Canada). The membrane was incubated with anti-MMP-2 antibody (Oncogene Science, Cambridge, MA) and subsequently with goat anti-mouse antibody conjugated with horseradish peroxidase (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA). ECL chemiluminescence detection (Amersham, Canada) was used to visualize the proteins.

Type I collagen degradation. Purified rat type I N-[propionate-2,3-^{3}H]propionylated-collagen (DuPont, Markham, ON) was dissolved in 0.01 N acetic acid and placed on plastic wells (96-well plates) at a concentration of 5 μg/cm^2 at 37°C. After 24 hours, the wells were rinsed with PBS and exposed to 10^5 PC-3 cells or 200 μl fresh CM *per* well for 24 hours at 37°C. Release of ^{3}H into the medium was determined as described above.

mRNA levels of matrix metalloproteinases. Total RNA was isolated from cancer cell lines with the phenol/chloroform extraction method (26). In brief, following exposure of cancer cell monolayers to the appropriate test conditions, cells were harvested by a 5-minute 0.5% trypsin/5.3 mM EDTA treatment. They were then lysed by vortexing in a chilled (0°C) solution of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$ and 0.5% Nonidet P-40. Cell debris was removed by a 2-minute 5000 rpm centrifugation. The RNA-containing supernatant was mixed 1:1 with 0.5% SDS in hot (65°C) phenol for 10 minutes and recovered by a 3-minute 6000 rpm centrifugation. The aqueous phase was mixed 1:1 with phenol/chloroform, vortexed for 5 minutes and recovered again by centrifugation. The RNA-containing aqueous phase was dissolved in 0.2 M sodium acetate pH 5.5 and 1:2 ethanol, and left overnight at -20°C. After standardizing the samples by spectrophotometry Northern and slot blot hybridizations were performed with cDNA probes for human interstitial collagenase (MMP-1), stromelysin 1 (MMP-3) and

TIMP-1, kindly provided by Dr. G.I. Goldberg (Washington University School of Medicine, St. Louis, MO) (27).

RESULTS

Degradation of Bone-Related and Bone Matrices. Human osteosarcoma cell lines, such as Saos-2 (17), conserve their osteoblastic phenotype (18) and secrete extracellular matrix components that form a matrix similar to osteoid. Due to these "osteoblast-like" properties, their matrices were used as substrates for tumor cell degradation. The matrices were metabolically radiolabelled with ^{3}H-proline, which is incorporated into *de novo* synthesized type I collagen. As shown in Table 1, PC-3 and SK-N-SH cells and their serum-free conditioned media significantly increased the release of ^{3}H compared to the control. These degradative effects were observed in both matrices, those derived from U-2 OS or Saos-2 cells.

In analogy to previous studies by Eilon and Mundy (4,9), we used metabolically-labelled non-viable rat fetal calvariae as degradation substrates for tumor cells. Degradation was assessed by the amount of ^{45}Ca released into the medium after exposure to either tumor cells or their conditioned medium. The data presented in Table 2 shows that PC-3 cells and their conditioned medium induced

significant increases in the release of ^{45}Ca into the medium, from as early as 24 hours and as late as 72 hours ($P<0.05$ *versus* serum-free medium).

Resorption pit assays allowed the morphological assessment of bone degradation by human bone-metastasizing cells. PC-3 cells induced formation of pits when placed on top of bovine bone slices for a period of 7 to 10 days. In sharp contrast to almost no resorption pits in unexposed bones, 23% ± 9 of the bone surface area showed resorption pits at day 10 (Table 3). Furthermore, since it has previously been shown that adhesion of PC-3 cells to bone-related substrates is stimulated by TGF-β1 (28), we examined if this growth factor could affect bone degradation by these cells. As shown in Table 3, TGF-β1 stimulated resorption pit formation by PC-3 cells at 4 ng/ml by 1.5 fold.

Role of Matrix Metalloproteinases in Direct Osteolysis by Tumor Cells. Enzymographic analysis of the conditioned medium from PC-3, SK-N-SH and Hs696 cells documented the presence of matrix metalloproteinases. More specifically, gelatinolytic and caseinolytic bands at M_r 90,000, 72,000 and 54,000, corresponding to 92- and 72-kDa type IV collagenases, and either interstitial collagenase or stromelysin, were detected in conditioned media from unstimulated cells (Figure 1). Since phorbol esters are known to stimulate the synthesis and release of matrix metalloproteinases by cancer cells (29), we challenged bone-metastasizing cells with TPA. Exposure of PC-3, SK-N-SH and Hs696 cells to 400

ng/ml TPA increased the release of these matrix metalloproteinases into the conditioned media (Figure 1). Since human HF-172 fibroblasts are known to respond to TPA by increasing matrix metalloproteinase release (29), they were used as a positive control. Conditioned medium obtained from SK-N-SH cells pre-stimulated with 100-400 ng/ml TPA, induced a 31% increase in degradation of osteosarcoma-derived matrices (from 766 ± 54 dpm to 1002 ± 41; $P<0.005$). This effect was similarly observed with PC-3 cells. Furthermore, matrix degradation by PC-3 cells was significantly inhibited in the presence of 50 mM 1,10-phenanthroline ($P<0.05$), a chelator of divalent cations known to inhibit matrix metalloproteinase activity (Figure 2) (30,31).

Modulation of matrix metalloproteinase release was also accomplished by exposure of Hs696 cells to purified human transforming growth factor β1. After exposure of these cells to 4-20 ng/ml TGF-β1, increased enzymatic release was observed at the M_r 92,000 and 61,000 gelatinolytic bands (Figure 3A). In contrast, under similar conditions, no changes were observed with the other bone-metastasizing cells. Northern and western blot studies demonstrated that this enzymatic induction was reflected in an increase in MMP-2 mRNA and protein, but not MMP-1 or stromelysin (Figures 3B and 3C).

To establish the capacity of bone-metastasizing cells to synthesize matrix metalloproteinases, total mRNA levels were assessed by northern blot hybridization

using human interstitial collagenase cDNA. We found that 20-400 ng/ml TPA induced an increase in mRNA levels of MMP-1 in both PC-3 and SK-N-SH cells. Again, human HF-172 fibroblasts were used as a positive control (Figure 4). We found no evidence of stromelysin expression by these three cell lines on northern and slot blot hybridization.

Since interstitial collagenases are enzymes known to degrade native type I collagen, we utilized ^{3}H-labelled purified rat type I collagen as substrate for degradation assays. PC-3 cells, as well as their conditioned medium, induced a 3-6 fold increase in type I collagen degradation over the control (Table 4) ($P<0.0001$). This degradation was significantly enhanced by a 24-hour pre-treatment of the cells with 100 ng/ml TPA after 48 hours exposure to PC-3 conditioned medium. Interestingly, conditioned medium consistently induced higher degradation than PC-3 cells ($P<0.0001$ at 24 and 48 hours).

DISCUSSION

The pathophysiology of bone metastasis, and in particular the mechanisms of osteolysis, are still not well understood (1,32). It is clear that osteoclast activation is often a predominant phenomenon in bone degradation (2). However, involvement of other cells, including the cancer cells, remains possible (1). Studies suggest that

tumor-associated macrophages and cancer cells may also directly produce local osteolysis (8,33). The present study addresses the direct degradative potential of three human bone-metastasizing cell lines and proposes that their matrix metalloproteinases are mediators of degradation.

Since osteosarcoma cells are characterized by the production of osteoid-like material containing type I collagen (34), we used extracellular matrices from osteosarcoma cell lines as substrates for degradation by tumor cells. Human bone-metastasizing cell lines degraded these non-mineralized matrices *in vitro*, as documented by release of metabolically incorporated radiolabelled proline. Additionally, our experiments with mineralized bone demonstrated that human prostate PC-3 adenocarcinoma cells were capable of inducing bone degradation. This was evident by their induction of radiolabelled calcium release from fetal calvariae and the formation of resorption pits in bovine bone slices. These approaches extend original reports by Eilon and Mundy (4,9), by demonstrating that bone-metastasizing human cancer cell lines are capable of directly inducing bone degradation. We observed demineralization produced by these tumor cells, the mechanisms of which have not been elucidated. Matrix demineralization must precede degradation of organic components such as type I collagen (35).

Matrix metalloproteinases are synthesized and secreted by many cells and have been extensively studied in the context of tumor cell invasion (36,37). Since type I collagen is the predominant protein in bone matrix (38), we analysed the metalloproteinase profile of the three human bone-metastasizing cells. Enzymography and hybridization studies showed that these cells produce and secrete gelatinase A and B and interstitial collagenase. This finding is important since interstitial collagenases and gelatinase B are known to degrade native type I collagen (39,40). To validate the functional significance of these findings, we challenged these cells with the phorbol ester TPA, known to stimulate the synthesis of matrix metalloproteinases through activation of protein kinase C (29). Such stimulation enhanced synthesis and secretion of these enzymes, as well as osteosarcoma-derived matrix degradation by these cells. Furthermore, PC-3 cells were capable of degrading purified native type I collagen, an effect also stimulated by TPA. These data, together with the abrogation of degradation by a matrix metalloproteinase inhibitor, allow us to propose that bone destruction by cancer cells is mediated, at least in part, by matrix metalloproteinases.

Transforming growth factor is known to inhibit matrix metalloproteinase production, and to stimulate synthesis of natural enzymatic inhibitors in fibroblasts and other normal cells (41). In our study, this growth factor induced an increase in matrix metalloproteinase release and gelatinase A synthesis by Hs696 cells. The importance of this finding relies on the fact that TGF-β1 is a predominant growth

factor embedded in bone matrix (42). Thus, stimulation of matrix metalloproteinase synthesis and release by TGF-β1, a less well known effect (43,44), constitutes a potential mechanism by which bone matrix degradation may encourage the aggressive phenotype of metastatic cells, and osteolysis in particular. Interestingly, significant upregulation of gelatinase A mRNA was not represented on enzymography. This finding emphasizes caution when interpreting studies in which only mRNA levels are considered. Studies are currently under way to further examine this observation.

Our data demonstrate that human cancer cells are capable of degrading bone-related and bone matrices without the participation of other cells, and that tumor-derived matrix metalloproteinases are involved in this destruction. We postulate that bone-metastasizing cells use this degradative machinery to cause osteolysis and release bone factors that may further stimulate the malignant phenotype of tumor cells. Our results implicate enzymes which may be therapeutically targeted in the prevention and treatment of bone metastasis.

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Table 1. *Degradation of osteoblast-like extracellular matrices by bone-metastasizing cell lines*

Conditions	U-2 OS-Derived Matrix		Saos-2-Derived Matrix	
	dpm	Percent Increase	dpm	Percent Increase
Serum-Free Medium	375 ± 17		373 ± 13	
PC-3 Cells	463 ± 27	24%[b]	533 ± 37	43%[b]
PC-3 Conditioned Medium	455 ± 18	21%[b]	503 ± 48	35%[b]
Serum-Free Medium	251 ± 24		673 ± 41	
SK-N-SH Cells	462 ± 19	84%[b]	766 ± 54	14%
SK-N-SH Conditioned Medium	401 ± 42	60%[b]	833 ± 37	24%[b]

[a] Each experimental value represents the percentage increase in ^3H release over control (serum-free medium) and determined from 8 individual replicates in each of two experiments.

[b] Statistically significant difference from serum-free medium (negative control).

Table 2. ^{45}*Ca-release from rat fetal calvariae in the presence of human bone-metastasizing prostate PC-3 adenocarcinoma cells and their conditioned medium*

Conditions	24 Hours[a]	72 Hours
PC-3 Cells	10 ± 1%[b]	15 ± 1%[b]
PC-3 Conditioned Medium	11 ± 1%[b]	13 ± 1%[b]

[a] Each experimental value represents the percentage increase in ^{45}Ca release over control (serum-free medium) and determined from 4 individual replicates.

[b] Statistically significant difference from serum-free medium (control).

Table 3. *Formation of resorption pits by human prostate PC-3 adenocarcinoma cells incubated on non-viable cortical bone*

Conditions	Exp. 1[a]	Exp. 2	Exp. 3
No Cells	2 ± 1%	5 ± 1%	2 ± 0%
PC-3 Cells	23 ± 9%[b]	25 ± 1%[b]	7 ± 0%[b]
PC-3 + TGF-β1 4 ng/ml	ND	ND	11 ± 1%[b]

[a] Each experimental value represents the percentage area of toluidine blue-stained areas in 10 microscopic fields. ND=not done.

[b] Statistically significant difference from negative control (no cells for PC-3 cells, and cells alone for TGF-β1).

Table 4. *Degradation of ^{3}H-type I collagen by human prostate PC-3 cells*

Conditions	24 Hours[a] (dpm)	48 Hours (dpm)
Serum-Free Medium	532 ± 31	384 ± 9
PC-3 Cells	1510 ± 36[b]	1625 ± 35
PC-3 Cells + TPA 100 ng/ml	1566 ± 48	1765 ± 56
PC-3 Conditioned Medium	1954 ± 41	2193 ± 40
PC-3 Cells + 100 ng/ml TPA-CM	2006 ± 51	2312 ± 38[c]

[a] Each experimental value represents ^{3}H release into the medium in disintegrations per minute and is expressed as mean ± SEM of 8 individual replicates.

[b] All values for cells and conditioned medum are significantly higher than those obtained for serum-free medium alone (P<0.0001).

[c] P<0.05 *versus* PC-3 conditioned medium.

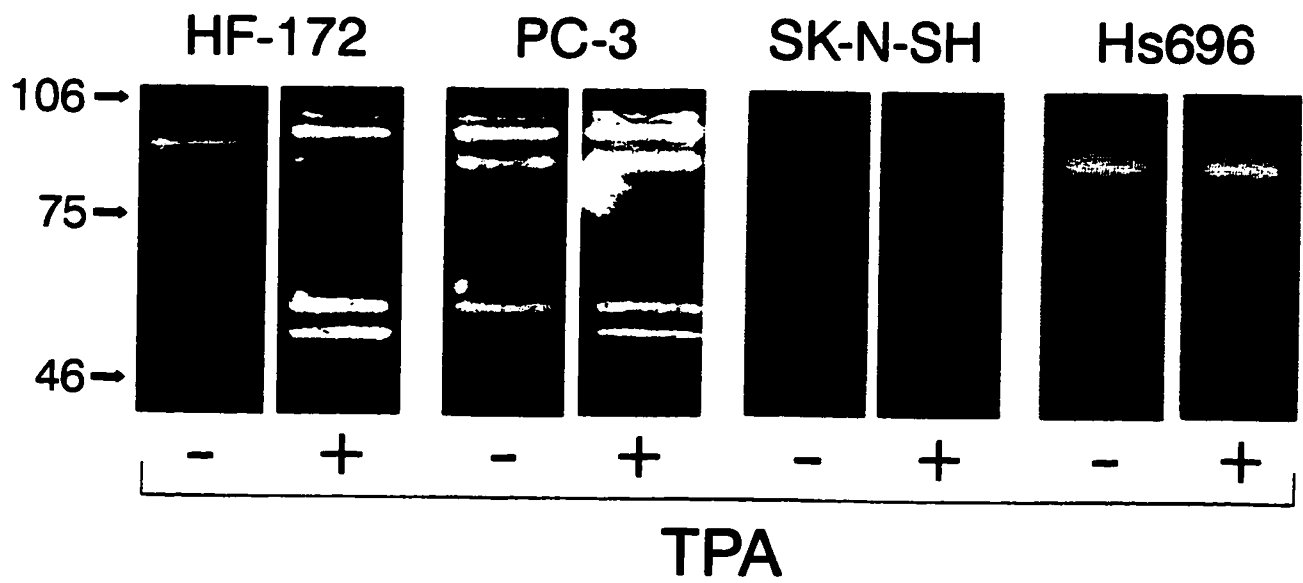
FIGURE LEGENDS

Fig. 1. Presence and stimulation of matrix metalloproteinases in conditioned media from human bone-metastasizing cells. Induction of enzymatic activity at M_r 92,000 and 52,000 by 400 ng/ml TPA was observed in the conditioned media of human HF-172 fibroblasts (positive control) and human bone-metastasizing prostate PC-3 adenocarcinoma, neuroblastoma SK-N-SH and adenocarcinoma Hs696 cells.

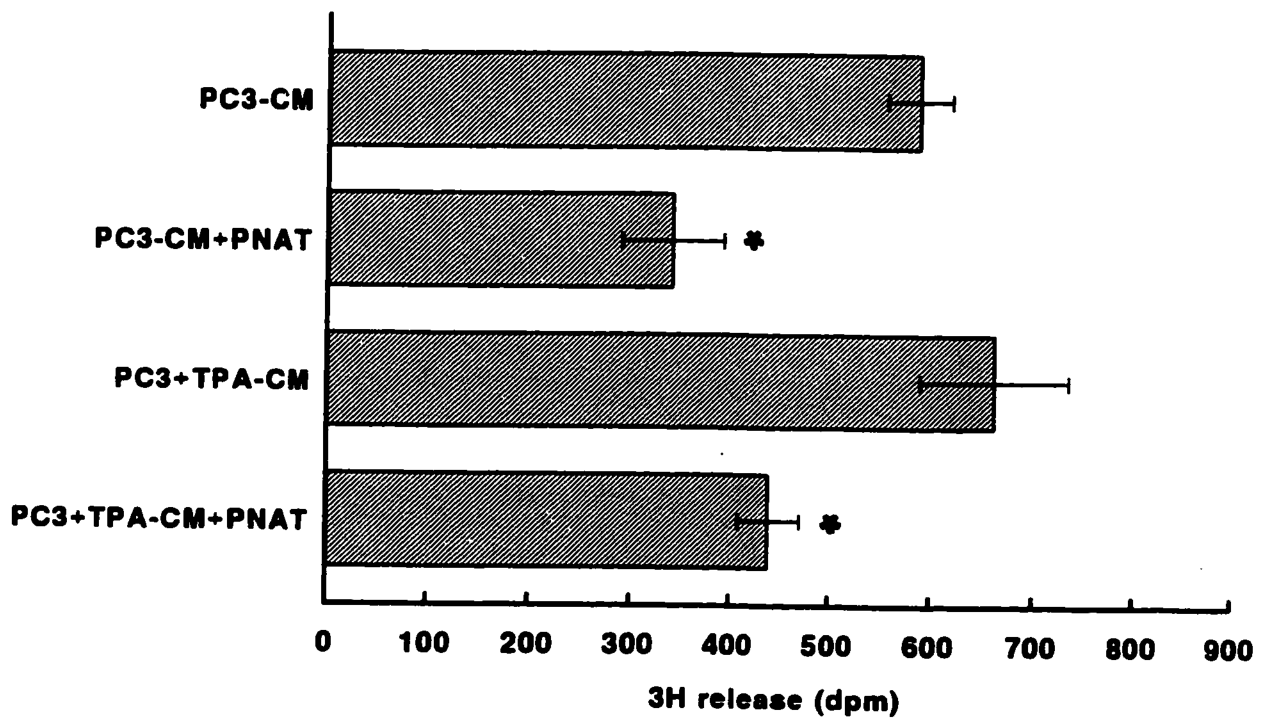
Fig. 2. Inhibition of matrix degradation by 1,10-phenanthroline. The degradative ability of conditioned medium collected from human prostate PC-3 adenocarcinoma cells was tested in the presence of 50 mM 1,10-phenanthroline. This chelator significantly decreased degradation of Saos-2-derived matrices by PC-3 conditioned medium ($P<0.005$ for PC3-CM and $P<0.05$ for PC3-CM from cells pretreated with TPA 400 ng/ml). Asterisks indicate a significant difference compared to control.

Fig. 3. Stimulation of matrix metalloproteinase release from Hs696 cells by TGF-β1. **(A)** Induction of enzymatic activity at M_r 94,000 and 54,000 by 4-20 ng/ml TGF-β1 was observed in the conditioned media of these cells. TPA was used as positive control. **(B)** Northern blot hybridization was performed with human MMP-1, MMP-2, MMP-9 and stromelysin cDNA probes after exposure of Hs696 cells to 20 ng/ml TGF-β1. Induction of enzymatic synthesis was observed only with MMP-2. Glycerol phosphate dehydrogenase (GAPD) expression was used as a standard for RNA loading. **(C)** Western blotting for MMP-2 under similar conditions showed an increase of this enzyme in the conditioned medium of Hs696 cells exposed to 20 ng/ml TGF-β1.

Fig. 4. Stimulation of MMP-1 synthesis in bone-metastasizing cells by TPA. Northern blot hybridization was performed with human MMP-1 cDNA probes after exposure of PC-3 and SK-N-SH cells to 100 ng/ml TPA. Induction of enzymatic synthesis was observed for MMP-1. Glycerol phosphate dehydrogenase (GAPD) expression was used as a standard for RNA loading.

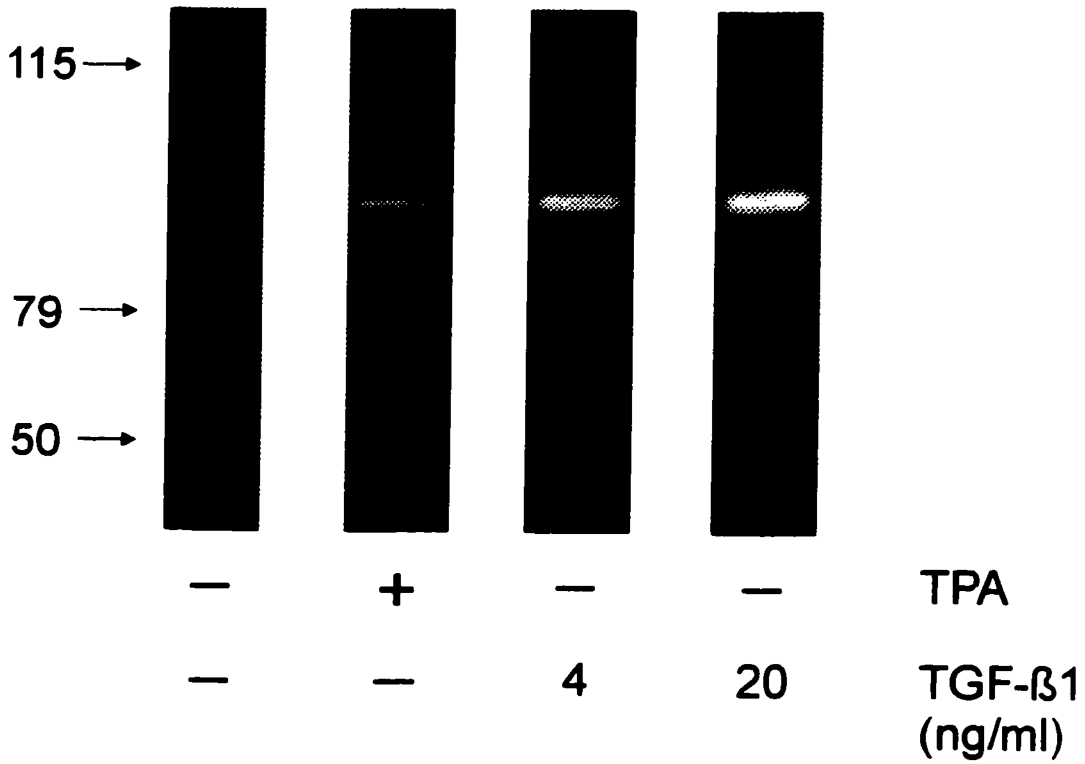


HF-172
PC-3
SK-N-SH
Hs696
106
75
46
-
+
-
+
-
+
-
+
TPA



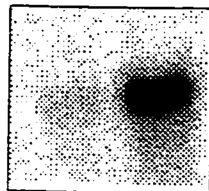
PC3-CM
PC3-CM+PNAT
PC3+TPA-CM
PC3+TPA-CM+PNAT
*
*
0
100
200
300
400
500
600
700
800
900
3H release (dpm)

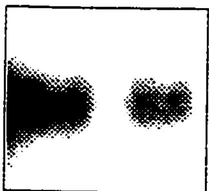
A



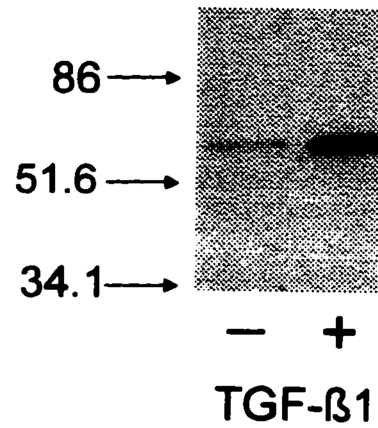
B

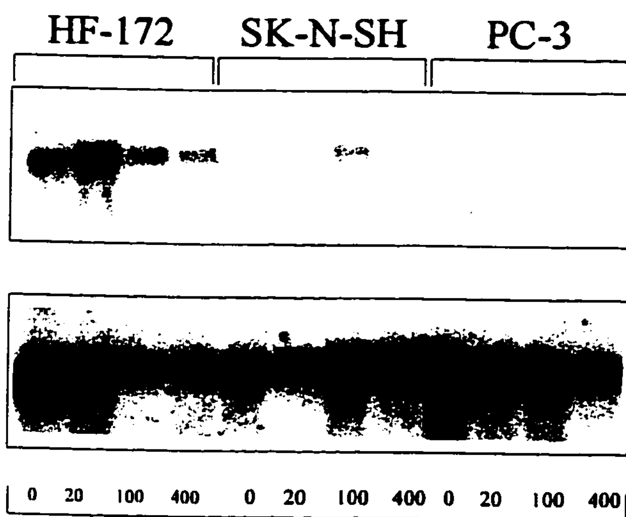
Hs696





C





MMP-1

GAPD

TPA
(ng/ml)

3. Additional Results and Discussion

This study confirms that the degradative abilities of cancer cells are shared by murine B16/F1 melanoma cells and the human bone-metastasizing cell lines described. These results can only be interpreted with caution: the fact that these cell lines were isolated from bone metastases does not necessarily mean that they preserve the phenotype that had the ability of spread to bones. Unfortunately, there are no "primary tumor" counterparts for these cells. Theoretically, it would be possible to obtain "primary" and "bone-metastasizing" counterparts by attempting spontaneous skeletal metastasis in nude mice. However, this has only been reported using in vitro selected PC-3 cells (Wang et al., 1991). Also, any findings resulting from these selection processes could be subject to bias by "clonal dominance" of metastatic cells (Kerbel, 1990).

One of the major limitations in interpreting the data that demonstrates ^{45}Ca release from bone, is that, strictly, these results only show that cancer cells are able to induce bone demineralization *with or without* concurrent collagen degradation. Thus, in an attempt to radiolabel the collagenous component of mineralized bones, I reproduced two protocols describing *in vitro* and *in vivo* labelling of rat fetal calvariae with ^{3}H-proline (Canalis et al., 1991a; Lerner et al., 1990a). *In vivo* labelling was achieved by subcutaneous injection of 150 μCi ^{3}H-proline in pregnant Sprague-Dawley rats on day 15 of gestation. Four days later calvariae were

prepared and exposed in serum-free conditions to 10^5 PC-3 or Hs696 cells and their conditioned medium for up to 96 hours. Aliquots of medium were collected and β-emissions determined. *In vitro* labelling was performed on calvariae from untreated pregnant rats, followed by incubation *in vitro* with 5 μCi/ml ^{3}H-proline for 24 hours. Bones were then devitalized and exposed to PC-3 or Hs696 cells and conditioned medium as described above. The results in Table 3 show that neither PC-3 nor Hs696 cells, nor their conditioned medium were able to consistently induce release of ^{3}H from these bones. Although total radioisotope uptake by calvariae was 20-fold higher with *in vitro* labelling than with *in vivo* labelled bones, the only significant induction of ^{3}H release was observed when *in vivo* labelled bones were exposed to Hs696 conditioned medium. The inconsistency of these results may be explained on technical grounds, since there is no evidence that adequate collagen labelling was achieved by either technique. Alternatively, it could be that tumor cells were not able, under the conditions used, to produce significant collagenolysis in these bone preparations.

Table 3. Degradation of ^{3}H-proline-labelled rat fetal calvariae by human PC-3 prostate and Hs696 adenocarcinoma cells

Conditions	*In vivo* labelling* (% release at 24 hrs)	*In vitro* labelling* (% release at 24 hrs)
Serum-free F12K	14 ± 2%	23 ± 1%
PC-3 cells	14 ± 1%	22 ± 1%
PC-3 CM	11 ± 3%	19 ± 1%
Serum-free DMEM	16 ± 0%	16 ± 1%
Hs696 cells	13 ± 1%	22 ± 1%**
Hs696 CM	22 ± 2%**	19 ± 1%**

* Three independent replicates used for each condition.
** $P<0.05$ vs. DMEM.

Characterization of other phenotypic features of the human bone-metastasizing cell lines used here, showed that bone-derived conditioned medium did not stimulate growth of these cells. In this context, and considering that the bone milieu contains many growth factors (Canalis et al., 1991b), I evaluated the ability of purified growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor I (IGF-I), fibroblast growth factor (FGF) and transforming growth factor β1 (TGF-β1) to stimulate release or activate tumor-derived matrix metalloproteinases. Thus, after exposing PC-3, SK-N-SH and Hs696 cell monolayers to phorbol esters or purified growth factors, their enzymatic profile on casein enzymography was assessed. As mentioned in the manuscript, a pattern compatible with activation of gelatinase B and increase in secreted interstitial collagenase was observed after exposing Hs696 adenocarcinoma cells to up to 20 ng/ml transforming growth factor β1. Exposure of these cell lines to other purified growth factors (0-100 ng/ml EGF, 0-5 half maximal units/ml PDGF, 0-200 ng/ml IGF-I, 0-200 ng/ml FGF) did not induce enzymographic changes in secreted MMP. Further, since TGF-β1 is known to be present in bone-derived conditioned medium (Centrella et al., 1985; Orr et al., 1990; Millar-Book et al., 1990), I exposed Hs696 to this medium to assess the inductive ability of this medium in enzymatic profile or degradative capacity of these cells. Bone-derived conditioned medium did not affect either.

In summary, these studies with human cells suggest that future *in vivo* studies should consider these cells as mediators of osteolysis together with osteoclasts, osteoblasts and tumor-associated macrophages. The experiments reported in this chapter demonstrate that these cells have the enzymatic machinery and are capable of inducing bone degradation. More *in vitro* studies are required to specifically address the issues around degradation of mineralized versus non-mineralized matrices. Perhaps future technical developments that use better bone substrates would allow this questions to be better addressed. Furthermore, the use of an adequate host for bone metastasis could be useful to test these observations *in vivo*.

E. DISCUSSION

1. Relevance

Bone metastases are frequently associated with osteolysis, a common radiological indicator of their presence. These lesions are associated with pain, pathological fractures, hypercalcemia and alterations in mechanical properties of the affected bones (Hipp et al., 1992; Body, 1992). Three cellular mediators of osteolysis have been proposed, namely, osteoclasts (Garrett, 1993), tumor-associated macrophages (Athanasou et al., 1992b; Athanasou et al., 1992b; Quinn et al., 1994b) and cancer cells (Eilon et al., 1983; Eilon et al., 1978; Mundy et al., 1978). Cancer cells can induce bone destruction and hypercalcemia systemically by secreting humoral factors which stimulate bone resorption, or locally by releasing paracrine mediators of bone degradation.

This thesis addresses the role of cancer cells in directly mediating bone matrix degradation. It stems from my initial observations in an animal model of experimental skeletal metastasis induced by murine B16/F1 melanoma cells. Histological examination of the bones two weeks after injection of tumor cells revealed that although osteolysis was evident, osteoclasts were proportionally decreased from resorptive surfaces, as determined by "routine" (hematoxylin/eosin) and histochemical (tartrate-resistant acid phosphatase) histomorphometry.

Quantitative analysis of these metastases confirmed these observations by demonstrating that the proportion of osteoclasts and osteoblasts were dramatically reduced in affected bones and that cancer cells were directly in contact with resorbing bone surfaces. Although these observations did not prove a direct role for cancer cells in osteolysis, they provided a basis to address the question of involvement of bone-metastasizing cancer cells *in vitro*. Thus, this project compiles my experimental delineation of interactions between tumor cells and the bone matrix that result in direct bone matrix degradation.

There are precedents for these ideas. Histologic observations of clinical and experimental bone metastases have shown cancer cells closely associated with resorptive surfaces of bone (Francini et al., 1993; Galasko, 1981) and concurrent bone resorption, which may be associated with disappearance of bone cells, including osteoclasts (Kanis et al., 1991). These reports have suggested that cancer cells may cause direct osteolysis, particularly at late stages of metastasis (Quinn et al., 1992; Galasko, 1981). In fact, Mundy *et al.* experimentally addressed the role of cancer cells in direct osteolysis, and obtained data demonstrating that human breast cancer cells and malignant histiocytes could, by themselves, induce release of radiolabelled calcium from fetal rat calvariae *in vitro* (Koeffler et al., 1978; Eilon et al., 1983; Mundy et al., 1978; Eilon et al., 1978). These findings, further reinforced by experimental observations in orthotopic (intramedullary) bone lesions by VX2 squamous carcinoma cells in rabbits, led Galasko to propose a "two-phase

hypothesis" of tumor-associated osteolysis: an *"osteoclast-dependent"* phase, during which osteoclasts are locally or systemically stimulated by tumor cells to proliferate, and destroy bone, followed by an *"osteoclast-independent"* phase in which osteoclasts disappear, and tumor cells and/or tumor-associated macrophages directly participate in the destructive process (Galasko, 1981a; Galasko, 1981b). Although this hypothesis has been further documented in other animal models, testing with human samples is troublesome since it would require a detailed histological time-course study, not a part of diagnostic protocols for bone metastases (Berrettoni et al., 1986).

Despite these observations, there is controversy concerning the ability of cancer cells to degrade bone directly (Quinn et al., 1994; Yoneda et al., 1994; Boyce, 1991; Rosol et al., 1992; Bataille et al., 1992; Garrett, 1993). For instance, it has been legitimately argued that the observation of cancer cells in direct contact with resorptive surfaces of bone does not necessarily prove that they are causing this resorption. Furthermore, considering that the evidence for a role of osteoclasts as mediators of osteolysis is strong, little attention has been placed on the potential role of other cells, such as tumor-associated macrophages and cancer cells, in local osteolysis. The *in vivo* descriptions of osteoclast-mediated resorption have been defined by a limited number of animal models. This limitation is only beginning to disappear as the use of cell types from different lineages becomes more widespread. Furthermore, it is necessary to address the direct role of tumor-

associated macrophages and tumor cells in bone degradation, and to answer important questions regarding the mechanisms used by these cells to degrade bone matrix. Currently, there is virtually no information concerning the specificity or predominance of cellular mechanisms of osteolysis according to tumor type. The existence of possible limiting steps in these processes and the efficacy of putative interventions to abrogate or reverse osteolysis have not been widely examined either.

This thesis, thus, addresses the role of cancer cells in directly degrading bone during progression of bone metastasis. The degradative interactions documented here presumably occur only after cancer cells have successfully lodged in the bones. For this reason, "bone-metastasizing" cells have been used. This implies that these cells have demonstrated their capacity to survive the blood environment, lodge and extravasate into the bone milieu and develop bone lesions. The findings reported here suggest that metastatic cells can directly degrade bone *in vivo* and bone-related matrices *in vitro*, by currently uncertain mechanisms, and implicate tumor-derived matrix metalloproteinases as likely mediators.

2. *In vivo* studies on matrix degradation by bone-metastasizing cells

Metastatic distribution after left ventricular injection of cancer cell lines

After left ventricular injection of cancer cells, the organs most affected by metastatic lesions are the bones, adrenal glands and ovaries (Arguello et al., 1991). This predominance was present with three different cell lines, and thus may be inherent to this particular method of injection. However, some cell lines, such as murine fibrosarcoma FS/L10 cells, display a well-defined neurotropism: in these experiments all animals injected with this fibrosarcoma cells had metastatic lesions in the central nervous system. Using these cells or murine melanoma cells, the distribution of skeletal secondary tumors predominantly affecting maxilla, mandible, femur and tibia was similar to that described in other experimental animals after intraarterial injection (Kjonniksen et al., 1990; Arguello et al., 1990; Arguello et al., 1988) and in human clinical cases (Galasko, 1981a; Easty et al., 1980a). The fact that there were no metastases in the kidneys, supports the notion that a good arterial supply and dense capillary network are not enough for the development of secondary lesions. The local organ microenvironment must also determine cancer cell viability and behaviour once vascular arrest occurs.

Tumor burden and cell proliferation in bone metastasis

In this phase of the project I tested the hypothesis that local factors associated with bone resorption can stimulate the proliferation of murine cancer cells. Using three murine tumor cell lines with dissimilar metastatic organ distribution, I determined growth and proliferation parameters in bone metastases *in vivo* and *in vitro* in the presence of bone-derived conditioned medium obtained from actively resorbing fetal mouse calvariae. This medium has been shown to contain transforming growth factor β and other growth factor activities (Centrella et al., 1985; Millar-Book et al., 1990).

The *in vivo* proliferative properties of the three murine cell lines, B16/F1, B16/F10 and FS/L10, as determined by ^{3}H-thymidine incorporation and tumor burden determination, were different for each cell type. These differences support a differential proliferative response of metastatic cancer cells to local tissue factors, as suggested by Kerbel *et al.* and thus, the idea that growth of metastatic cells is affected by organ-specific microenvironmental factors (Kerbel, 1992). For instance, it is possible that these cells could display different cell cycling properties, either inherently or dominated by their microenvironment (organ-derived growth inhibitory factors).

Evaluation of skeletal tumor burden by B16/F10 cells showed that this cell line displayed the highest bone involvement of the cell lines studied. In contrast, this same cell line showed, in bone, proliferative labelling indexes (^{3}H-thymidine incorporation) that were considerably lower than those of the other two cell lines. To explore these *in vivo* observations, I evaluated tumor cell proliferation *in vitro*, using the same proliferative marker as employed *in vivo*, after exposure of the tumor cell lines to factors associated with bone resorption. Only B16/F10 cells showed an increase in ^{3}H-thymidine uptake when exposed to up to 40% bone-derived conditioned medium. As mentioned above, this same cell line displayed *in vivo* the lowest ^{3}H-thymidine uptake in bone. Metastatic lesions in bone by these cells were, in contrast, the largest when compared with other cell lines. These observations were analyzed in a correlation test for all three cell lines: high ^{3}H-thymidine uptake in the presence of bone-derived factors *in vitro* correlated positively with high bone tumor burden (r=0.97) and negatively with ^{3}H-thymidine incorporation *in vivo* (r=-0.97). These seemingly contradictory findings, could be explained by the notion that large bone tumors may halt rapid proliferation after trophic limitations become overwhelming for tumor cell viability. This speculation could be, in the future, tested by studying the proliferative features of metastatic tumor cells in time-course experiments. These studies attempting to compare and correlate *in vivo* and *in vitro* proliferative behaviour of tumor cells could be largely inconclusive, unless a large number of cell types is used, thus increasing the statistical strength of the observed correlations.

The complexity of animal models hinders analysis of all possible factors involved in the development of skeletal metastasis, at this time. In this study we chose two kinetic parameters that are quantifiable and represent tumor cell proliferation and growth. Increased tumor burden of B16/F10 cells in bone could be explained in association with phenomena related to tumor-specific cell dynamics or host response to this particular cell line. It is possible that, once B16/F10 cells arrest in bones, they initially proliferate rapidly with the support of bone-derived factors, until hypoxia or other local limiting factors, such as exhaustion of bone factors, hinder further tumor cell proliferation.

In summary, these experiments suggest that the *in vitro* proliferative behaviour of metastatic cells may, to some degree, reflect their *in vivo* proliferation. This may be important since understanding of host-tumor cell interactions, particularly those related to the effects of the bone microenvironment in tumor growth, may lead to a better understanding of the pathogenesis of bone metastasis and to potential therapeutic advances against human skeletal metastasis. Within the context of this thesis, however, these findings were not further explored. Nevertheless, these observations together with previous studies (Orr et al., 1993; Orr et al., 1995), were encouraging since they further argued in favour of the possibility that bone microenvironmental factors locally affect the malignant phenotype of tumor cells.

Osteolysis in bone metastasis: histomorphometry

Quantitative analysis of experimental metastasis in bone is considered essential to document morphological changes associated with tumor invasion. Initial histomorphometric studies of bone metastasis by murine B16/F1 melanoma cells injected intraarterially into syngeneic mice showed significant bone matrix degradation associated with local invasion by tumor cells, virtual eradication of osteoclasts and osteoblasts, and close association between the presence of tumor cells and bone resorption surfaces. These findings corresponded with those described for the "osteoclast-independent" phase previously reported in experimental and clinical studies (Berrettoni et al., 1986; Galasko, 1981). These findings demonstrated that local osteolysis occurs in this animal model, and suggested direct involvement of murine melanoma cells in local bone destruction. Although it has been argued that the presence of tumor cells on resorption pits does not necessarily mean that these cells are responsible for resorption (Boyce, 1991), it is striking that we observed a marked proportional decrease in osteoclasts, the cells that are the main mediators of osteolysis. Furthermore, the time course study in which metastatic lesions were analyzed at 0,5, 10 and 15 days after injection of tumor cells revealed that the histological evolution of bone metastasis by B16/F10 cells was not associated with an early increase in osteoclasts, as documented with other tumors, such as rabbit VX2 carcinoma and rat W256 mammary tumor cell line (Galasko, 1982; Krempien et al., 1993). This observation

was confirmed using tartrate-resistant acid phosphatase activity as a more sensitive osteoclast detection system.

These findings allowed me to conclude that in this experimental model: (1) bone metastases were associated with significant local loss of bone matrix, and that (2) this bone loss was possibly due to direct bone degradation by tumor cells, since there was an almost complete disappearance of osteoclasts from the bone surfaces and since most resorptive areas were closely associated with tumor cells.

3. In vitro studies on matrix degradation by bone-metastasizing cells

The tumor cells used for *in vitro* analysis included a murine melanoma cell line known to metastasize to bone with high efficiency following intraarterial injection, and three human cell lines obtained from bone metastatic lesions. Prostate PC-3 cells have been widely studied and found to secrete gelatinase A (Stearns et al., 1993) and display plasminogen activator activity (Gaylis et al., 1989). The degradative abilities of SK-N-SH neuroblastoma and Hs696 adenocarcinoma cells have not been previously reported.

Since osteosarcomas are of osteoblastic origin and typically produce osteoid rich in type I collagen (Matsuura et al., 1996), I used matrices derived from

osteosarcoma cell lines as *in vitro* substrates for metastatic melanoma cells (Mundy et al., 1995). I have predominantly used Saos-2-derived matrices, since of all human osteosarcoma cell lines available, these cells distinctively exhibit elevated alkaline phosphatase activity and are highly committed to the osteoblastic phenotype (Rifas et al., 1994).

The data showed that murine B16/F1, human PC-3, SK-N-SH and Hs696 bone-metastasizing cells, and their conditioned medium degraded osteoid-like matrices. The ability of these cells to degrade mineralized matrices was tested on three different bone preparations, including osteosarcoma-derived matrices exposed to β-glycerophosphate, fetal rat calvariae and bovine bone slices. These experiments indicated that these cells degrade mineralized bone matrices. Previously, Mundy *et al.* (Mundy et al., 1978; Eilon et al., 1978) demonstrated that human cell lines released radioactive calcium from fetal rat calvariae. The data presented here with murine melanoma and bone-metastasizing cells, using osteoid-like and bone substrates, confirm their findings.

In degradation assays using human osteosarcoma-derived matrices, release of radiolabel induced by cancer cells directly apposed and attached to these matrices was frequently higher than that induced by conditioned medium from these cells. These differences in degradation between the cells and their medium, presumably containing soluble factors secreted by these cells, may be due to the

presence of proteases or other factors in the cell membranes (Trabandt et al., 1990; Whitelock et al., 1991). In fact, membrane-associated matrix metalloproteinases have been identified and recently characterized as mediators and activators of proteolytic activity (Takino et al., 1995).

The mechanisms by which cancer cells induce demineralization of bone matrix are not known. Osteoclasts degrade bone in a "sealed zone" by a combined action of membrane proton-channels and proteinases, resulting in extracellular acidification, matrix demineralization and degradation (Everts et al., 1992; Gluck, 1992). These specialized cells contain a variety of lysosomal acid hydrolases such as acid phosphatases, carboxylester-hydrolases, glycosidases, peptidases, sulphatases, and non-specific esterases. These are released into the extracellular milieu and play an active role in resorption of the bone matrix. Other enzymes such as lysosomal acid cysteine-proteinases (cathepsins B, C, L and N) and perhaps neutral matrix metalloproteinases (interstitial collagenases, stromelysin and others) may also participate in the degradation of type I collagen, the main organic component of the matrix. The end result is the formation of *resorption pits* (Howship's lacunae) with a depth of 60-100 μm. The presence of similar mechanisms in cancer cells has not been reported. I speculate that cancer cells may locally acidify their immediate microenvironment by other yet undefined mechanisms, in a less efficient way than osteoclasts. Additionally, although cancer cells express type I collagen-binding integrins (Kostenuik et al., 1996; Albelda,

1993), it is unlikely that they will produce focal degradation analogous to the $\alpha_v\beta_3$-mediated osteoclastic osteolysis (Teti et al., 1991), since they are clearly not specialized to do this, in contrast to osteoclasts that are.

4. Role of matrix metalloproteinases in matrix degradation by bone-metastasizing cells

Tumor-derived enzymes that degrade extracellular matrix components have been described in the context of tumor cell invasion and metastasis (Matrisian, 1992a; Stetler-Stevenson et al., 1993a). Furthermore, type I collagen, the most predominant protein in the bone matrix (Vaananen, 1993), can be degraded by interstitial collagenases including MMP-1, MMP-2 (gelatinase A) and MMP-13 (collagenase-3) (Knauper et al., 1996; Aimes et al., 1995). These enzymes belong to the family of matrix metalloproteinases which are secreted by cells in a latent form and subsequently activated by extracellular molecules such as plasmin and stromelysin (Nagase et al., 1991). Upon activation, and in the presence of divalent cations, these enzymes degrade collagens and other matrix substrates or may be inactivated by specific inhibitors (DeClerck et al., 1994).

The role of matrix metalloproteinases in the metastatic process has gained strong experimental support during the last few years. In fact, various *in vitro* and

in vivo studies have documented the need for active matrix metalloproteinases in tumor invasion and metastasis. Specifically, evidence has been gathered on the role of 72- and 92-kDa type IV collagenases (MMP-2 and MMP-9, respectively) and stromelysin (MMP-3) in basement membrane degradation, as a step required by malignant epithelial cells to initiate invasion.

Interstitial collagenases belong to the family of MMPs known to degrade type I collagen (Mignatti et al., 1993). In this study, a role for MMPs in tumor-induced osteolysis was demonstrated *in vitro* by: (a) inhibition of tumor-induced matrix degradation by a chelator of divalent cations, (b) degradation of purified type I collagen as a specific substrate for MMPs, and (c) stimulation of degradation by the phorbol ester TPA. Enzymography confirmed that this was associated with increased release of active matrix metalloproteinases by tumor cells. Thus, we conclude that tumor-derived MMPs are mediators of direct osteolysis by murine melanoma cells. That they are not the single predominant mediators, is suggested by the *in vivo* studies with TIMP-1 overexpressing B16/F10 transfectants, that did not show clear differences in skeletal histomorphometry when compared with non-overexpressing counterparts. Further, *in vitro* inhibition of matrix degradation was not complete, which suggests the presence of other non-cation-dependent degradative mechanisms.

Matrix metalloproteinase production by tumor cells

To document the production of MMPs by bone-metastasizing cells, I used commercially available human cell lines derived from bone metastases, including PC-3 prostate adenocarcinoma, SK-N-SH neuroblastoma and Hs696 adenocarcinoma of unknown histogenesis. Additionally, following the histomorphometric observations with B16/F1 cells (described above), the production of matrix metalloproteinases by these cells was assessed. Enzymographic studies established that these cells synthesize and secrete matrix metalloproteinases. These findings confirm reports by Huijzer *et al.* (Huijzer et al., 1995) of production of MMP-9 and MMP-2 by B16/F1 cells. The validity of casein as an appropriate substrate for MMP-1 was confirmed by two approaches: (a) RNA hybridization studies with MMP-1 cDNA probes (see below), and (b) enzymography of immunoprecipitated human synovial fibroblast MMP-1, which shows a caseinolytic band indistinguishable from that observed in cancer-derived conditioned media (immunoprecipitates kindly provided by Albert Agro and Dr. Carl Richards).

Enzymography is a sensitive technique to detect enzymatic activity with a specific substrate embedded in the resolving gel. This technique may be complemented by mRNA studies for the specific enzymes under scrutiny.

Enzymography and mRNA levels have been shown to correlate well in studies with human cancer cell lines (Brown et al., 1990).

Matrix metalloproteinase synthesis by tumor cells: mRNA studies

The availability of human matrix metalloproteinase cDNA probes opened the possibility of studying gene expression of human MMP-1, MMP-3 (stromelysin) and TIMP-1 (tissue inhibitor of metalloproteinases type 1) in bone-metastasizing human cancer cells. MMP-1, as well as MMP-3 can degrade casein and display similar gel migration properties (Mignatti et al., 1993). Thus, gene expression studies not only allow establishing specifically that the caseinolytic bands observed correspond to MMP-1 rather than MMP-3, but also to verify if a given modulatory effect on MMP-1 has a transcriptional component (see below).

Murine B16/F1 cells did not display, on northern blot hybridization, expression of any of the matrix metalloproteinases or their inhibitors. This may be interpreted on technical grounds: the cDNA probes used were human in nature. Although the homologies between human and murine matrix metalloproteinases are over 50% (Dioszegi et al., 1995), some investigators have found similar incompatibilities when using the human probes for murine samples (L. Matrisian and G. Goldberg, personal communications).

In contrast to murine cells, northern and slot blot hybridizations with cDNA probes for human MMP-1, MMP-3 and TIMP-1 demonstrated the presence of corresponding mRNA transcripts in all cell lines. Thus, I confirmed the enzymographic observation that the human cell lines under study synthesize matrix metalloproteinases. These results complement other studies showing that cancer cells produce a wide variety of matrix metalloproteinases (Mackay et al., 1992).

Matrix metalloproteinase modulation by extracellular factors

I tested the idea that homeostatic bone factors and other matrix components may upregulate tumor-mediated bone matrix degradation. For this purpose, I studied the production of matrix metalloproteinases by cell lines originally obtained from metastatic bone tumors, and other experimental bone-metastasizing cells. It was hypothesized that the exposure of these cells to purified growth factors or other constituents of bone would upregulate bone matrix degradation as determined by quantitative degradation of bone and related matrices, enzymography and MMP-1 transcript expression.

Transforming growth factor β is a 25,000 Da homodimer, composed of 112 amino acid monomers, of which there are 5 isoforms, known as transforming growth factors β1, β2, β3, β4 and β5, with up to 70% sequence homology between them (Sporn et al., 1990). In bone, transforming growth factor β is produced by

osteoblasts and stimulates osteoblast activity in an autocrine fashion, while also inhibiting osteoclastic resorption (Mundy et al., 1990). In sharp contrast with previous observations that TGF-β will on its own not have any effect on synthesis and release of MMP-1 (Edwards et al., 1987), or will inhibit this enzyme, I found that this growth factor alone could induce the release of matrix metalloproteinases by Hs696 adenocarcinoma cells. The mechanism by which this occurs has not been elucidated. Interestingly, northern blot hybridization demonstrated increase in the mRNA levels of MMP-2 only. I speculate that this selective regulation may be related, in Hs696 cells, to specific modifications in the signal transduction pathways that control matrix metalloproteinase expression. In this regard, it is known that the promoter sequences of most matrix metalloproteinases contain a TGF-β1 inhibitory element (TIE) (Matrisian, 1994; Matrisian et al., 1992). It is possible that in Hs696 cells regulation of this element could be altered. This issue may, in the future, be addressed by detailed study of MMP gene expression in these cells. Other investigators have recently reported similar inductive functions by TGF-β1, especially in metastatic *versus* non-metastatic cells (Sehgal et al., 1996; Shimizu et al., 1996) and discussed the potential implications of these responses in the progression towards metastasis (Kerbel, 1992).

Matrix metalloproteinases in matrix degradation: chelating agents

Most investigations of MMPs have been performed studying their gene expression by densitometric assessment of specific mRNA levels and/or by semi-quantitative enzymographic activity on an artificial substrate. However, as MMP activity is regulated by complex intracellular and extracellular processes, analysis must ultimately be confronted in functional studies of matrix degradation (Ponta et al., 1994). With this in mind, *in vitro* degradation assays were applied.

Chelating agents have been used to determine the dependence of divalent cations for MMP activity (Ossowski, 1992). In this study. I used 1,10-phenanthroline, a chelator of divalent cations, known to inhibit matrix metalloproteinases by removing zinc atoms required for their activity (Springman et al., 1995). In this study, 1,10-phenanthroline inhibited matrix degradation by murine B16/F1 cells *in vitro*. The inhibitory activity of this chelator in assays with human cells lines was not clear, perhaps suggesting that other proteinases may be important in mediating their degradative activity. This could be easily addressed by using more specific MMP inhibitors, such as purified TIMPs, or by testing the role of other enzymes with use of their specific inhibitors in functional assays. Although useful, the major disadvantage of using chelators as MMP inhibitors is that they may inhibit other cation-requiring cellular functions, including membrane-associated proteins such as integrins.

Matrix metalloproteinases in matrix degradation: phorbol esters

The original description by Brinckerhoff *et al.* (Brinckerhoff et al., 1979) of phorbol ester modulation of collagenase release from human fibroblasts, lead to further work exploring this modulation in cancer cells. In perspective, there are two main disadvantages for the use of this method as a model to study modulation of matrix metalloproteinases: (1) TPA is an artificial agent, and (2) it activates protein kinase C (PKC), thus triggering multiple downstream effects on gene transcription (Brinckerhoff et al., 1979). This has elicited concerns about the relevance and/or specificity of the experimental observations.

In this project, TPA was used as a stimulator of matrix metalloproteinase activity in cancer cells. I demonstrated that in the cells used, this agent induced the secretion and synthesis of these enzymes. This has been described in many other tumor cell lines (Mackay et al., 1992). In these studies, TPA also induced degradation of osteoblast-like extracellular matrices and purified type I collagen by bone-metastasizing cell lines. These results were used as evidence that, since by inducing these enzymes degradation is affected, matrix metalloproteinases were involved in matrix degradation. TPA could, however, induce these degradative effects indirectly, by triggering other cellular functions involved in tumor cell invasion (Pazin et al., 1992; Kohn et al., 1995). Because of these additional

mechanistic implications, I used type I collagen as a specific matrix metalloproteinase substrate.

Matrix metalloproteinases in matrix degradation: type I collagen degradation

Native type I collagen is a triple helical protein that is degraded at specific sites by interstitial collagenases. These enzymes, upon activation, and in the presence of divalent cations such as calcium, split the $\alpha 1$ and $\alpha 2$ chains of native helical type I collagen at specific sites on residue 775, located about one-fourth of the distance from the COOH-terminus (Pauli et al., 1988; Fields, 1991). Thus, after cleavage, two types of fragments are formed, TC^A (3/4) and TC^B (1/4) (Stricklin et al., 1988). These fragments can further be degraded by other matrix metalloproteinases and plasmin (Pauli et al., 1988; Overall et al., 1987).

In initial experiments, I used human fibroblast-derived ^{14}C-collagen type I as substrate for MMP-1. Radiolabelled type I collagen was purified by selective salt precipitation (Overall et al., 1987). Tumor-derived conditioned media was incubated with known amounts of this substrate in the presence of a Ca^{++}-containing buffer for 24 hours. The integrity of the $\alpha 1$ and $\alpha 2$ chains was then evaluated by resolution in 7.5% SDS-PAGE and autoradiography. This allowed me to specifically establish the activity of MMP-1 in a quantitative manner. I was able to reproducibly demonstrate type I collagen degradation by commercially available purified leech

collagenase as a positive control (Rigbi et al., 1987). However, it was not possible to confirm the presence of ***active*** MMP-1 in the conditioned media of cancer cells with this approach. This negative observation could be explained by: (a) presence of MMP-1/TIMP-1 complexes in the conditioned medium; in fact, TIMP-1 is also produced by cancer cells and is inducible by phorbol esters, (b) presence of MMP-1 at low concentrations not sufficient to induce detectable ^{14}C-type I collagen degradation, (c) further degradation of 3/4-1/4 cleavage fragments by other enzymes, thus making them not autoradiographically evident, or (d) presence of MMP-1 in a latent form.

Final identification of the matrix metalloproteinases responsible for tumor-derived bone matrix degradation was not achieved in this project. The unavailability of antibodies capable of neutralizing specific activities has hindered this task. The antibodies currently available are useful to detect specific matrix metalloproteinases with western blots or immunohistochemistry, but not to inhibit their activity. Since these antibodies became available only at the end of this project, their preliminary use was not included in this thesis. Using these specific probes, it will be important to address in the future, the modulatory effects by phorbol esters or growth factors on the synthesis or secretion of matrix metalloproteinases by bone-metastasizing tumor cells.

Intracardiac injection of murine TIMP-1 transfectants

Tissue inhibitors of metalloproteinases (TIMPs) are peptides that specifically and non-covalently bind to matrix metalloproteinases, blocking their proteolytic effect. Accordingly, the overall collagenolytic activity is presumed to be determined by a balance between enzymatic production and activation, and the synthesis and secretion of TIMPs by tumor cells and/or host cells (DeClerck et al., 1994). In fact, investigators have demonstrated that TIMP-mediated inhibition of MMPs may inhibit invasion and metastasis both *in vitro* and *in vivo* (DeClerck et al., 1992; Khokha, 1994; DeClerck et al., 1994; Khokha et al., 1995).

Since TIMP-1 is expressed in bones beginning in early development (Flenniken et al., 1990) and this seems to coincide with TGF-β expression, I evaluated if TIMP-1 expression in the tumor cells would change the metastatic behaviour of cancer cells in bone. Taking advantage of the availability in Dr. Rama Khokha's laboratory of genetically engineered murine tumor cell clones transfected with TIMP-1 cDNA and overexpressing secreted TIMP-1 (Khokha et al., 1992), I assessed *in vivo* the role of TIMP-1 on the development of experimental skeletal metastasis. The metastatic behaviour of these transfectants in bone were histomorphometrically analyzed. These murine B16/F10 TIMP-1 transfectants display less tumorigenicity and ability to develop experimental lung metastases than their parental cells (Khokha, 1994). The fact that the results were inconclusive,

proved that TIMPs are obviously not the only determinants of osteolysis by cancer cells, and that other mechanisms must be involved. Further, it supports the multicellular origin of metastasis-associated local osteolysis.

5. Conclusions and future directions

Based on the findings described above, I propose that cancer cells may directly degrade osteoid and mineralized matrix by mechanisms involving the release of matrix metalloproteinases, including interstitial collagenases and gelatinase A. Potential mechanism(s) by which cancer cells could demineralize and then, degrade bone collagen remain unknown and are, at this stage, speculative at best. These studies on osteolysis may explain why antiosteoclastic agents may not completely prevent or treat metastasis-associated local osteolysis (Mundy, 1991). Specifically, if tumor cells are also responsible for direct bone degradation, then treatments which inhibit osteoclastic activity, such as those with bisphosphonates, should be complemented by inhibitors of tumor cell-mediated degradation. In light of these observations and those of other investigators, I propose that once metastatic cancer cells arrest at the bone microenvironment, various cell-cell and cell-matrix interactions may result in bone destruction: (1) cancer cells may secrete cytokines and/or growth factors which activate osteoclasts and osteoblasts, (2) tumor-associated macrophages may induce direct bone matrix

degradation, by yet unknown mechanisms, but perhaps analogous to osteoclasts, originated from the same lineage, and (3) cancer cells may degrade osteoid and mineralized bone matrix by the production of active matrix metalloproteinases and other unknown mechanisms. In turn, resulting bone degradation products, such as matrix-embedded growth factors or fibrillar protein fragments, may be released and further stimulate osteolysis by enhancing cancer cell production and/or activation of lytic enzymes. These mechanisms need not be exclusive, and may predominate at different stages during the temporo-spatial development of skeletal metastasis. Moreover, it is likely that metastatic cells from different histologic lineages will primarily utilize one of these mechanisms.

I consider that a role for matrix metalloproteinases in direct tumor-induced osteolysis is important since these enzymes may constitute a limiting step for this process. Moreover, since they are therapeutically accessible (Dodwell, 1993; Davies et al., 1993; Chapman et al., 1993; Beszant et al., 1993; Bird et al., 1994), novel approaches could be derived. For instance, since at diagnosis, about one third of patients with breast or prostate cancer have tumor cells "seeded" in the bone marrow (Lindemann et al., 1992; Beiske et al., 1992), "antiosteolytic" therapy could aim at preventing or delaying development of skeletal metastasis. Bisphosphonates have been used to block osteoclastic bone resorption (Fleisch, 1991). Perhaps recently described matrix metalloproteinase inhibitors may provide a novel alternative to prevent metastasis-associated local osteolysis. In clinical

trials for primary and secondary neoplasias, matrix metalloproteinase inhibitors such as batimastat, have been shown to affect growth and metastatic ability of human cancer cells *in vivo* (Sledge, Jr. et al., 1995).

This project provides further pathogenetic insight into the clinically relevant process of bone metastasis and proposes a new avenue in prophylactic or therapeutic strategies with potential beneficial effects in patients with bone metastasis. It demonstrates direct bone matrix degradation by tumor cells and characterizes this effect as mediated, at least in part, by MMPs. These observations strengthen the concept that osteoclasts, tumor-associated macrophages *and* cancer cells directly participate in local bone matrix degradation associated with bone metastasis.

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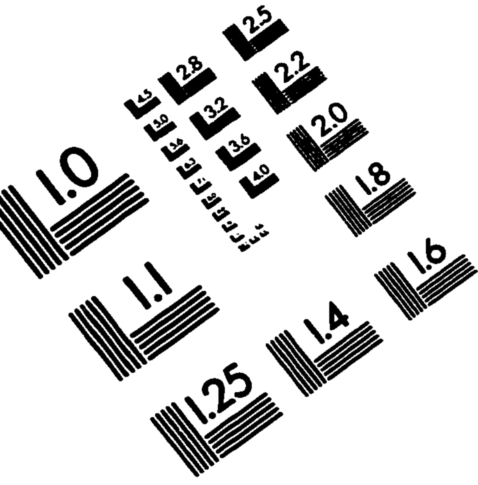
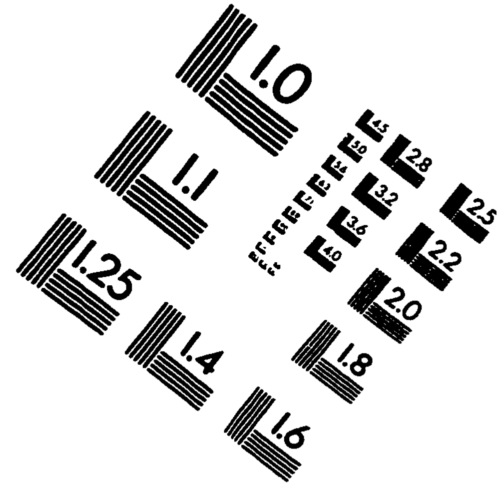
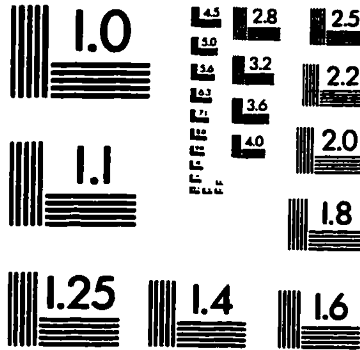
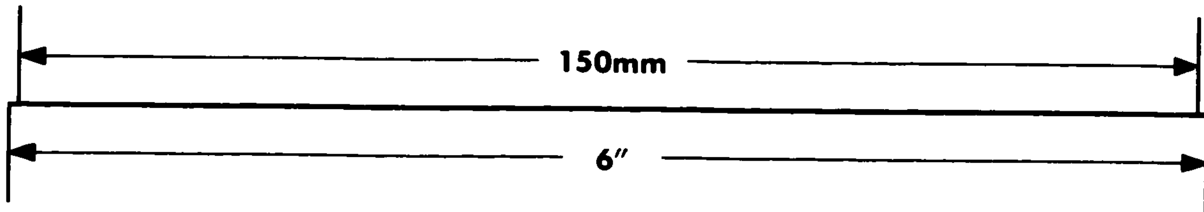
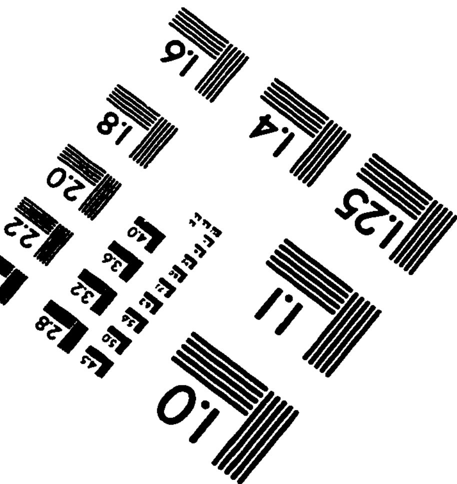
IMAGE EVALUATION TEST TARGET (QA-3)











APPLIED IMAGE . Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

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