Clin. Exp. Metastasis, 1992, **10**, 411–418

Stimulation of bone resorption results in a selective increase in the growth rate of spontaneously metastatic Walker 256 cancer cells in bone

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(Received 10 April 1992; accepted 22 June 1992)

To test the hypothesis that bone metastasis is related to the rate of bone remodeling, we have examined the effect of enhanced bone resorption on the growth of spontaneously metastatic Walker 256 (W256) cancer cells. Bone resorption was stimulated in male Fischer rats by injecting Rice H-500 Leydig tumor cells subcutaneously. The resorptive response of the skeleton was confirmed in a pilot study by evaluating parameters of bone morphometry after 4, 7 and 10 days of tumor burden. The distal femoral epiphyses had 35 ± 10% more osteoclast surface, 83 ± 11% less osteoblast surface, and 46 ± 5% less trabecular bone after 10 days of tumor burden, compared to non-tumor-bearing controls. To evaluate the effect of Leydig tumor-induced bone resorption on the growth response of W256 cells, 20 rats were injected intramuscularly with 2×10^7 W256 cells, and 20 rats were vehicle-injected. Two days later, 10 rats from each group were injected subcutaneously with Leydig tumor cells. Twelve days after W256/vehicle injection, rats were injected with [^{3}H]thymidine, killed 2 h later, and their femurs, liver, lungs and kidneys were processed for histology. In rats injected with Leydig tumor cells only, enhanced bone resorption was confirmed by a 40 ± 4% increase in serum calcium concentration, a 48 ± 8% decrease in trabecular bone content, and a 72 ± 15% decrease in osteoblast surface, compared with non-tumor-bearing rats. Metastatic W256 cells adjacent to trabecular bone in Leydig tumor-bearing rats had a 56 ± 18% greater relative [^{3}H]thymidine labeling index (TdR) than did W256 cells in the bones of non-Leydig tumor-bearing rats. The TdRs of W256 cells in the liver, lungs, and kidneys were not affected by Leydig tumor burden. In this model, enhanced bone resorption was associated with the selective growth promotion of metastatic W256 cells in bone, suggesting the existence of a bone-derived factor which is mitogenic to W256 cells.

Keywords: bone metastasis, growth, hypercalcemia, osteolysis

Introduction

The ability of malignant cancer cells to spread from a primary site to distant locations is a function of various interactions between tumor cells and the host. The growth of metastatic cells has been shown to be influenced by the environment within the tissues that they colonize [1–3]. Human malignancies often have distinct, predictable patterns of hematogenous spread which are independent of the first capillary bed encountered [4]. For example, carcinomas of the breast, prostate, lung, kidney and thyroid have a propensity to metastasize to bone, an event which is often associated

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with osteolysis and hypercalcemia [5, 6]. While the skeleton is rarely the exclusive target of metastatic cells, the extent and frequency of its involvement would not be predicted by its modest blood supply, estimated at 5–10% of cardiac output [7].

Disseminated tumor cells may interact with the skeleton in ways which facilitate bone metastasis. For example, in patients with known malignancy who present with Paget's disease, a disorder of increased bone remodeling, it has been reported that the Pagetic lesions are often the first sites of skeletal metastasis [8, 9]. Such observations have suggested the hypothesis that the rate of bone remodeling regulates the development of local bone metastases. This hypothesis has been supported by our observations *in vitro* that the growth of metastatic Walker 256 (W256) tumor cells is influenced by bone resorption rates. The W256 rat tumor is of monocytoid origin [10], metastasizes aggressively to bone, and causes extensive osteolysis [11, 12, 12a]. The ability of conditioned media from bone organ cultures to stimulate the growth and chemotaxis of W256 cells correlated directly with the extent to which bones had undergone resorption [13, 14]. *In vivo*, we have demonstrated that within the rat skeleton, spontaneously metastatic W256 cells located adjacent to trabecular bone had significantly greater uptake of [^{3}H]thymidine, an index of growth rate, than did W256 cells located >50 μm from bone [12a].

To determine if the *in vivo* growth rate of W256 cells in bone would respond positively to stimulated bone resorption, we have examined the incorporation of [^{3}H]thymidine by W256 cells in bone and in other organs from rats bearing the Rice H-500 Leydig cell tumor. When transplanted subcutaneously, the non-metastasizing Leydig tumor cell line has been shown to stimulate bone resorption in rats. The effects of 13–19 days of tumor burden have been reported to include increased osteoclast number or activity [15–18], decreased formation surface [15, 16, 18], decreased trabecular bone content [15, 17, 18], and hypercalcemia [15, 16, 18, 19]. These effects on the skeleton have recently been attributed to the production and secretion by Leydig tumor cells of parathyroid hormone-related protein (PTHrP) [20, 21], a polypeptide which has been shown to cause similar systemic skeletal responses when directly injected into rats [22, 23]. By subcutaneously transplanting Leydig tumor cells, we have stimulated bone resorption and examined the subsequent behavior of metastatic W256 cells. We report that the stimulation of bone resorption is associated with a selective increase in the growth of W256 cells in bone, but not in other organs.

Materials and methods

Animals and cell lines

Male Fischer 344 rats (150–175 g) were purchased from Charles River Laboratories (St Constant, Quebec, Canada) and were accommodated under a 12 h light/dark cycle, with Rodent chow and water supplied *ad libitum*. The Rice H-500 Leydig cell tumor line was obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) and was passaged several times subcutaneously. For experiments, subcutaneous tumors from 2 to 3 rats were excised, and the capsules and necrotic tumor tissue dissected and removed. The viable tumor tissue was finely minced, and the explants from the different hosts were combined and suspended in Hanks' balanced salt solution (Gibco, Grand Island, NY, USA). The suspension was triturated vigorously through a 16-guage needle to further dissaggregate the tumor cells. In each experiment, animals from various groups were injected from the same suspension of cells, which were triturated again between each injection to ensure constant Leydig tumor cell concentrations. Injections were alternated between groups such that each group of animals received cells with similar durations of explant from the host. The properties, maintenance, and isolation of the W256 cells (Flow Laboratories) have been described previously in detail [24].

Effects of Leydig tumor burden on bone morphology

A pilot study was conducted in order to establish the effects of Leydig tumor burden on parameters of bone morphology over time. Twenty male Fischer 344 rats (150–175 g) were injected subcutaneously in the left flank with 0.5 ml of minced Leydig tumor suspension, using a 16-guage needle. Ten rats were injected with 0.5 ml of Hanks' solution alone. Groups of tumor-bearing rats were killed on days 4, 7 and 10. The control rats were killed on day 10 after vehicle inoculation. The left and right femurs were removed, dissected free of soft tissue, and prepared for histology.

Effects of Leydig tumor burden on metastatic behavior of W256 cells

Twenty male Fischer 344 rats (150–175 g) were injected with 2×10^7 W256 cells in 200 μl of

Hanks' solution in the left upper thigh muscle. Twenty additional animals were vehicle-injected with 200 μl of Hanks' balanced salt solution alone. Two days later, 10 rats from each of the two groups were injected subcutaneously in the left flank with 0.5 ml of minced Leydig tumor suspended in Hanks' solution. The 10 control rats from each group received vehicle injections of 0.5 ml Hanks' solution. Twelve days after the W256 cell/vehicle inoculations, all animals were injected with 2 μCi of [^{3}H]thymidine (sp. act. 81 μCi/mmol; Dupont, Canada), anesthetized with a solution containing 4 mg of xylazine and 20 mg of ketamine (in 200 μl of 0.9% saline) and killed 2 h later by exsanguination. Terminal blood samples were obtained, and the intramuscular W256 tumor masses were excised and measured in three dimensions. The liver, both femurs, lungs and kidneys were removed and processed for histology.

Preparation of tissues

The right and left femurs were removed, dissected free of soft tissue, and fixed in dimethylsulphoxide (DMSO) which contained 10% formalin. A section of primary W256 tumor was also taken from the W256 tumor-bearing rats and was fixed in 10% formalin/DMSO. The lungs, liver, kidneys and a sample of the primary W256 tumor were removed from all animals and were fixed in 10% formalin. They were then processed for histology and embedded in paraffin wax. Tissue sections were cut with a Sorvall JB-4A microtome (Dupont, Newtown, CT, USA). Three-μm sections of the soft tissue, including the primary W256 tumors, were prepared and used for autoradiography. After 24 h in fixative the distal third of each femur from all animals was bisected longitudinally and fixed for an additional 24 h. For the experiment employing both W256 and Leydig tumors, the undecalcified right femur (contralateral to the site of W256 cell injection) and the primary W256 tumor were dehydrated in graded ethanol and impregnated with JB-4 embedding medium (Analychem, Canada). For the study using the Leydig tumor only, both right and left femurs were prepared.

A 2 μm section was cut from both the primary W256 tumor and the right femur of each W256 tumor-bearing animal, and was attached to the same slide. One set of slides, each containing bone and (where applicable) primary W256 tumor, was stained with 1% Toluidene Blue (pH 6.9), and was used for morphometric analysis. Another set of slides, prepared only for the study using both tumors, was used to compare the autoradiographic labeling of primary W256 cells to that of metastatic W256 cells in bone, liver, lung and kidney.

Bone morphometry

Bone morphometry was performed on 2 μm undecalcified longitudinal sections of the distal third of the femur. For the study using only the Leydig tumor, the distal epiphysis of both femurs were analysed. For the study utilizing both tumors, the right femur was chosen for analysis. The left femur was excluded as it was immediately adjacent to the primary W256 tumor site, and could be influenced by invasive as well as metastatic phenomena. A Mertz graticule [25] was employed under oil immersion light microscopy (×1000 magnification) to obtain measurements for bone morphometry. The area of the epiphysis located from 300 to 400 μm from the distal epiphyseal growth plate was chosen for analysis, based on observations from previous morphometric analysis that this region was particularly susceptible to tumor-induced osteolysis [12a]. The percentage of each microscopic field occupied by mineralized trabecular bone was determined by the percentage of point measurements which were superimposed on trabecular bone. This value was then divided by the total area occupied by trabecular bone and marrow to determine the percentage of total tissue area occupied by trabecular bone (trabecular bone content) [26]. The percentage of trabecular bone surfaces occupied by osteoblasts, osteoclasts, or osteoid was determined by recording their presence or absence where a trabecular bone surface was intercepted by the grid of the Mertz graticule. Osteoblast surface, osteoclast surface, and osteoid surface were calculated as the length of trabecular bone surfaces occupied by osteoblasts, osteoclasts, or osteoid, respectively, divided by the total trabecular bone length, the quotient of which was multiplied by 100 [26].

Measurement of W256 tumor cell growth rate

Slides of plastic-embedded primary W256 tumor with bone sections, and slides of paraffin-embedded primary W256 tumor with liver, lung and kidney were coated with NTB2 autoradiographic emulsion (Kodak, Canada). The coated slides of the paraffin-embedded tissue were developed after 7 days of exposure to the emulsion, and slides of the plastic-embedded tissue were developed after 14 days of exposure at 4°C. Different exposure periods were required due to the

differences in the density of the embedding media. After development, slides were fixed and then stained with either 1% Toluidene Blue (plastic-embedded tissue) or hematoxylin and eosin (paraffin-embedded tissues). Cellular uptake of [^{3}H]thymidine was quantified by the number of grains visible above 100 W256 cells from each section of primary W256 tumor, bone, lung, liver, and kidney. The number of grains required for a cell to be considered *positive* was calculated as the mean number of grains, plus 1 S.D., over 100 primary tumor cells. This criterion consistently included 10–20% of primary tumor cells. The percentage of labeled cells in each metastatic site was divided by the percentage of labeled cells in the same animal's primary tumor to obtain a relative thymidine labeling index (TdR) [27].

Serum chemistry

Tail vein blood samples were obtained from each rat, and the samples were used for the determination of serum calcium, serum alkaline phosphatase (ALPase) activity (a marker or osteoblast activity) [28], and serum tartrate-resistant acid phosphatase (TRAPase) activity (a marker of osteoclast activity) [29]. Serum alkaline phosphatase and tartrate-resistant acid phosphatase were determined with assay kits from Sigma (St Louis, MO, USA). Calcium samples were measured by a discrete dry chemistry analyser (Kodak Ectachem 700 XR) using reflectance spectrophotometry (Eastman Kodak, Rochester, NY, USA).

Statistical analysis

For the statistical comparison of parameters of bone morphometry in Leydig tumor-bearing animals over time, the groups with different durations of tumor burden were compared to non-tumor-bearing controls using Student's *t*-test and a 95% level of confidence. All data are presented as the mean ± standard error of the mean (S.E.M).

Results

Effects of Leydig tumor burden over time

Compared to non-tumor-bearing controls, trabecular bone content in Leydig tumor-bearing animals transiently increased (13 ± 4%) after 4 days of tumor burden, and then decreased progressively (Figure 1). After 10 days of Leydig tumor burden, trabecular bone content was 46 ± 5% less than that seen in non-tumor-bearing rats. The biphasic influence of Leydig tumor burden on trabecular bone content was accompanied by changes in the relative distribution of bone cells lining the trabecular surfaces. In association with the early increase in trabecular bone content, there was a

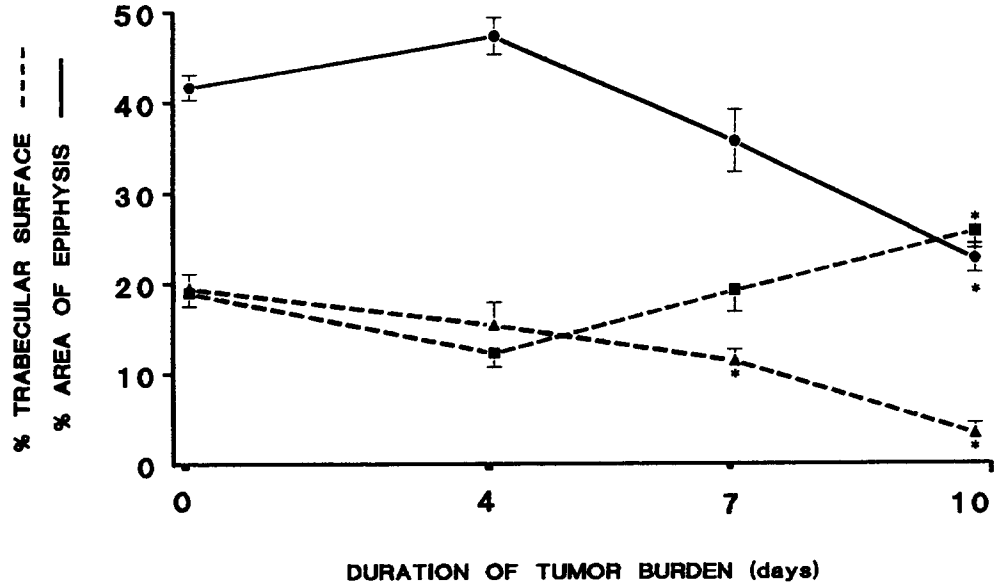

Figure 1. The effects of Leydig cell tumor burden on parameters of bone morphometry. Rats were injected subcutaneously with Leydig tumor cells, and their femurs were dissected 4, 7 or 10 days later. The percentage of the total area of the distal epiphysis occupied by trabecular bone (●), and the percentage of total trabecular bone surface length occupied by osteoblasts (▲), and by osteoclasts (■) was analysed. *Significantly different from day 0, $P < 0.05$. Mean ± S.E.M.

35 ± 10% decrease in osteoclast surface compared to non-tumor-bearing controls. Osteoclast surface subsequently increased sharply, and was 35 ± 8% higher after 10 days of Leydig tumor burden compared to non-tumor-bearing controls. Osteoblast surface decreased progressively with the duration of tumor burden, and was significantly lower in rats with 7 days (42 ± 9%) and 10 days (83 ± 11%) of Leydig tumor burden than in non-tumor-bearing rats (Figure 1).

These results suggested that 10 days of Leydig tumor burden was an effective means by which to increase bone remodeling in rats. We subsequently sought to evaluate the response of spontaneously metastatic W256 cells to the skeletal alterations induced by 10 days of Leydig tumor burden.

Effects of Leydig tumor burden on metastatic behavior of W256 cells

Biochemical parameters of bone remodeling. In this experiment, 10 days of Leydig tumor burden was associated with a 40 ± 4% increase in serum calcium concentration as compared to non-tumor-bearing controls (Table 1). Serum calcium concentration was unaffected by W256 tumor burden alone, but was 19 ± 3% greater in rats bearing both tumors as compared to normal controls. Leydig tumor burden and W256 tumor burden were both associated with decreases in serum ALPase activity (60 ± 5% and 53 ± 5%, respectively) as compared to non-tumor-bearing controls. A similar decrease (56 ± 5%) was observed in animals bearing both tumors, as compared to non-tumor-bearing controls (Table 1). The Leydig and W256 tumors alone were both associated with non-significant increases in serum TRAPase activity. Animals bearing both tumors had significantly greater serum TRAPase activity (25 ± 7% greater) as compared to non-tumor-bearing controls (Table 1).

Bone morphometry. There was a decrease in mineralized trabecular bone in rats bearing either the W256 or the Leydig cell tumors (Table 2). Compared to non-tumor-bearing controls, rats with Leydig cell tumor burden had 48 ± 8% less trabecular bone content, and rats with W256 tumor burden had 35 ± 10% less trabecular bone. Animals bearing both tumors had 61 ± 10% less trabecular bone than non-tumor-bearing rats, and had significantly less trabecular bone than did rats bearing either tumor alone.

The distribution of the cells lining trabecular bone surfaces was altered in animals bearing either tumor (Table 2). The osteoclast surface was not significantly altered in rats bearing either tumor alone, but rats bearing both tumors had a significantly greater osteoclast surface than did rats bearing the W256 tumor alone (62 ± 11% increase) or rats bearing no tumor (36 ± 8% increase). Leydig cell tumor burden was associated with a 72 ± 15% decrease in the osteoblast surface, as compared to non-tumor-bearing controls. Animals bearing the W256 tumor had 94 ± 18% less osteoblast surface than did non-tumor-bearing controls. The osteoblast surface in rats bearing both tumors was virtually non-existent. The percentage of trabecular bone surface occupied by osteoid was 43 ± 8% decreased in Leydig tumor-bearing rats. Osteoid surfaces were virtually non-existent in rats bearing the W256 tumor.

[^{3}H]Thymidine uptake by W256 cells. The incorporation of [^{3}H]thymidine by metastatic W256 cells was compared to that of W256 cells from each rat's primary tumor to obtain a relative labeling index (TdR) for W256 cells at each metastatic site. In all organs, W256 cells had greater [^{3}H]thymidine uptake than did W256 cells from the primary tumor. In the liver, lungs and kidneys,

Table 1. The effects of Leydig tumor burden on serum-derived parameters of bone remodeling in control and W256 tumor-bearing rats (mean ± S.E.M)

Condition	Calcium (mmol/l)	ALPase (U/ml)	TRAPase (U/ml)
−Leydig			
Control	2.7 ± 0.1	11.5 ± 0.5	1.4 ± 0.1
+W256	2.7 ± 0.3	5.4 ± 0.4[a]	1.7 ± 0.2
+Leydig			
Control	3.8 ± 0.2[a]	4.6 ± 0.3[a]	1.6 ± 0.2
+W256	3.2 ± 0.1[a,b]	5.1 ± 0.3[a]	1.7 ± 0.1[a]

[a]Significantly greater than non-Leydig-bearing control rats ($P < 0.01$).
[b]Significantly greater than non-Leydig-bearing W256 tumor-bearing rats ($P < 0.05$).

Table 2. The effects of Leydig cell tumor burden on parameters of bone morphometry in control and W256 tumor-bearing rats (mean ± S.E.M)

Condition		% of total trabecular bone surface		
	TBC[a] (%)	Osteoclast surface	Osteoblast surface	Osteoid surface
−Leydig				
Control	39 ± 3	25 ± 2	32 ± 4	21 ± 2
+W256	25 ± 3[b]	21 ± 3	2 ± 1[b]	0 ± 0[b]
+Leydig				
Control	20 ± 2[b]	30 ± 2	9 ± 2[b]	12 ± 3[b]
+W256	15 ± 3[b,c]	34 ± 3[c]	0 ± 0[b,d]	0 ± 0[b,d]

[a]Trabecular bone content.
[b]Significantly less than non-Leydig tumor-/non-W256 tumor-bearing rats ($P < 0.05$).
[c]Significantly different than W256 tumor-bearing, non-Leydig tumor-bearing rats ($P < 0.05$).
[d]Significantly less than Leydig tumor-bearing, non-W256 tumor-bearing rats ($P < 0.0001$).

the relative labeling of W256 cells was the same in Leydig tumor-bearing rats as in non-Leydig tumor-bearing rats. While Leydig tumor burden had no effect on [^{3}H]thymidine uptake by W256 cells in non-osseous organs, the relative labeling of W256 cells in the bones of Leydig tumor-bearing rats was 56 ± 18% greater than that observed in the bones of non-Leydig tumor-bearing rats (Table 3).

Discussion

It has been previously demonstrated *in vitro* that Walker 256 carcinosarcoma cells exhibit chemotactic and mitogenic responses to the products of bone resorption, and that the magnitude of these responses correlate highly with the extent of resorption which had occurred in calvarial bone cultures [13, 14]. We thus examined the possibility that the stimulation of bone resorption *in vivo* might result in the stimulation of growth of spontaneously metastatic W256 cells in bone. The results indicate that Leydig tumor-stimulated bone resorption was associated with a 56 ± 18% greater TdR of metastatic tumor cells in bone, while the TdRs of W256 cells localized in non-osseous organs were unaffected.

Table 3. The effect of Leydig tumor burden on [^{3}H]thymidine uptake by metastatic W256 cells relative to [^{3}H]thymidine uptake by W256 cells from the primary tumor. Values (mean ± S.E.M) represent the labeling index of metastatic W256 cells in individual organs divided by the labeling index of primary W256 cells from each rat (see Materials and Methods for calculation of labeling index)

Metastatic target organ	Relative labeling index (TdR) of W256 cells	
	Non-Leydig-bearing	Leydig-bearing
Bone	4.3 ± 0.8	6.7 ± 1.2[a]
Liver	4.6 ± 1.0	4.7 ± 1.1
Lung	5.2 ± 1.4	5.8 ± 0.8
Kidney	6.5 ± 0.7	6.2 ± 0.8

[a]Significantly greater than TdR of W256 cells in the bone of non-Leydig tumor-bearing rats ($P < 0.05$).

The effects of Leydig tumor burden on parameters of bone morphometry observed here are consistent with those reported previously. We first examined the skeletal response to increasing duration of tumor burden. From days 4 to 10 of tumor burden, observed increases in osteoclast surface and decreased osteoblast surfaces could account for the progressive decrease in trabecular bone content. In a study designed to evaluate W256 cell responses to 10 days of Leydig tumor burden, we found that Leydig tumor burden was also associated with hypercalcemia, with significantly decreased ALPase activity, and with non-significantly increased TRAPase activity. Changes in these serum-derived markers of osteoblast and osteoclast activity, respectively, were associated with decreased osteoblast surface and with non-significantly increased osteoclast surface. In animals bearing both tumors, osteoclast surface was significantly elevated over those rats bearing either the W256 tumor alone or bearing no tumor. This observation may suggest that the local effects of metastatic W256 tumor cells and the systemic effects of the primary Leydig tumor might have

additive effects on osteoclast proliferation. Such a phenomenon would explain the more dramatic osteolysis observed in rats bearing both tumors versus those bearing either tumor alone.

The observed changes in parameters of skeletal morphology and metabolism were associated with a selective stimulation of W256 cell growth rate in bone but not in other organs. The unique relationship between bone resorption rate and the growth rate of tumor cells in bone provides further evidence that the skeleton might provide a favorable soil for the growth of disseminated tumor cells. This evidence includes the observation that the products of resorbing bone are mitogenic to W256 cells. It has also been shown *in vitro* that purified transforming growth factor-β (TGF-β), a polypeptide found in the greatest quantity in the skeleton, stimulates the growth of W256 cells in a dose-dependent manner [13]. TGF-β is a component of organic bone matrix [30], and is synthesized and secreted by osteoblasts [31]. Its release from fetal rat calvaria cultures has been shown to correlate highly with bone resorption rates, as well as with the growth response of W256 cells. The addition of anti-TGF-β antibody to bone-derived conditioned media largely inhibited the W256 cell growth response, suggesting that bone-derived TGF-β, and perhaps other bone-derived proteins, stimulate W256 cell growth in a manner related to bone remodeling [13]. These *in vitro* observations, and the *in vivo* observations reported here, support the existence of a servomechanism for tumor cell growth stimulation, whereby the increased bone resorption which often accompanies certain malignancies might enhance the release of a putative matrix-derived mitogen for metastatic tumor cells. The existence of such a feedback mechanism is consistent with the previously-reported observation that 92% of breast cancer metastases in bone were positive for PTHrP, compared to only 17% of metastases from non-osseous sites [32].

The present study supports the concept that the process of bone remodeling might release into the bone microenvironment factors which selectively stimulate tumor cell growth rate. The putative mitogen and its source remain to be elucidated, but may include TGF-β or other matrix- or osteoblast-derived growth factors.

Acknowledgement

Supported by a grant from the Medical Research Council of Canada (MA-10409).

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