Pergamon

European Journal of Cancer Vol. 33, No. 6, pp. 918–925, 1997

Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(97)00513-8

Original Paper

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

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

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

We examined the osteolytic ability of metastatic cells and the role of tumour matrix metalloproteinases (MMPs) in bone degradation. The histomorphometry of experimental bone metastases of 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 the presence of TPA-inducible M_r 90000, 84000 and 64000 gelatinolytic, and 54000 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. © **1997 Published by Elsevier Science Ltd.**

Key words: metastasis, bone, matrix metalloproteinases, osteolysis, melanoma

Eur J Cancer, Vol. 33, No. 6, pp. 918–925, 1997

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 hypercalcaemia [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 in cancer-associated hypercalcaemic syndromes [3]. In these conditions, 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 this phenomenon occurs 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 of murine melanoma cells. Our findings suggest that tumour cells directly degrade bone matrix. This study shows that B16/F1 cells degrade bone-related and mineralised bone matrices *in vitro*, and provides evidence that tumour-derived MMPs mediate this phenomenon.

MATERIALS AND METHODS

Intra-arterial 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. Intra-arterial injections followed procedures by Arguello and associates [11]. Subconfluent

Correspondence to G. Singh, Director of Research, Hamilton Regional Cancer Centre, 699 Concession Street, Hamilton, Ontario, Canada L8V 5C2.
Received 29 May 1996; revised 28 Oct. 1996; accepted 6 Nov. 1996.

cells were harvested with 0.01 M ethylenediaminetetraacetic 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, Quebec, Canada) were anaesthetised by intramuscular injection of 32 mg/kg ketamine hydrochloride (Rogarsetic™, Rogar/STB, London, Ontario, Canada) and 32 mg/kg xylazine hydrochloride (AnaSed™, Lloyd Laboratories, Shenandoah, Iowa, U.S.A.). 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. From the suspension, 0.1 ml containing 10^5 cells was injected within 20 seconds and followed by skin suture. After the 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 eiphysis of each mouse was selected for morphometric analysis, since it is a common site for metastases after intra-arterial 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 h and plastic-embedded without decalcification. Staining for TRAP was accomplished by incubation of the sections at 37°C for 75 min with a solution containing acetate solution, naphthol AS-BI phosphoric acid and tartrate solution (Sigma Diagnostics, St. Louis, Missouri, U.S.A.). 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, Maryland, U.S.A) in MEM supplemented with FBS and antibiotics, plus 5 μCi/ml ^{3}H-proline (NEN, Boston, Massachusetts, U.S.A.) on 96-well plates for 5 days. Cells were lysed with 20 mM NH_4OH at 37°C for 20 min 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 MEM under serum-free conditions for 24 h. Release of ^{3}H was determined 24 h later by collecting 50 μl aliquots of medium into scintillation fluid (Ready Safe™, Beckman, Fullerton, California, U.S.A.) and counted in a Beckman LS1801 liquid scintillation beta counter (Beckman, Irvine, California, U.S.A.). Each value was expressed as the mean ± SEM of eight replicates. Mineralisation of Saos-2 matrices was obtained by incubation with 10 mM β-glycerophosphate (Sigma) 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 nM 1,10-phenanthroline (Sigma) 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 MEM and frozen at −20°C to induce death of bone cells. After 24 h, calvariae were thawed, rinsed and incubated with 10^5 B16/F1 cells or 1 ml fresh B16/F1-CM medium per bone for 24 h at 37°C. ^{45}Ca release was determined as described above. Each value was expressed as the mean ± SEM of four replicates.

Resorption pit assay

Longitudinal bovine cortical bone slices from the femoral midshaft were prepared according to Chambers and associates [16]. Slices (5 × 5 × 2 mm) were cut with an Isomet low-speed diamond-edged saw (Buehler Instruments, Evanston, Illinois, U.S.A.), cleaned by ultrasonication for 30 min, washed in ethanol, dried and stored. After the slices were presoaked with serum-free medium, 10^5 B16/F1 cells were placed on to 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 H_2O for 6 h, the slices were fixed with 10% formaldehyde or 2.5% glutaraldehyde in PBS. Slices were stained with 1% toluidine blue for light microscopy according to Ritchie and associates [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, Ontario, Canada) was dissolved in 0.01 N acetic acid and placed on plastic wells at a concentration of 5 μg/cm^2 at 37°C. After 24 h, the wells were rinsed with PBS and exposed to 10^5 B16/F1 cells or 200 μl fresh CM per well for 24 h 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 MEM. 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 h. Samples of B16/F1 conditioned medium were collected and centrifuged to remove cell debris. Protein-standardised samples were resolved at 4°C in 10% SDS–PAGE containing 1 mg/ml gelatin or casein (Sigma) [18]. Incubation with 2.5% Triton X-100 for 30 min 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 h at 37°C. Gels were stained with 2.5% Coomassie Blue for 30 min, 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, Virginia, U.S.A.).

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 intra-arterially 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 approximately a 70% decrease in bone matrix compared with tumour-negative bones (9 ± 2% versus 28 ± 4%, respectively; $P < 0.0026$, 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 assess the presence of osteoclasts in bone metastasis further, TRAP activity was detected on plastic-embedded femurs recovered 5, 10 and 15 days postinjection 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 mineralised in the presence of 10 mM β-glycerophosphate [14], TPA-inducible degradation by B16/F1 cells and their CM was observed (Table 2).

Table 1. Histomorphometric evaluation of B16/F1 bone metastasis

	Tumour positive* $n = 7$	Tumour negative $n = 7$	*t*-test
	Mean ± SEM	Mean ± SEM	*P* value
Area†			
Bone	9 ± 2%	28 ± 4%	<0.0026
Bone marrow	1 ± 1%	72 ± 4%	<0.0001
Tumour	90 ± 2%		
Cells on trabecular surfaces‡			
Osteoblasts	0 ± 0%	30 ± 6%	<0.0001
Osteoclasts	1 ± 1%	9 ± 3%	<0.0480
Tumour cells	71 ± 8%		
Resting	28 ± 7%	61 ± 7%	<0.0060
Resorption pits§			
Osteoclasts	4 ± 4%	100 ± 0%	<0.0001
Tumour cells	96 ± 4%		

*Seven C57BL/6 mice were injected intra-arterially 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 tumours were used as controls. †Values represent areas of bone, bone marrow and tumour, expressed as percentages of total area examined. ‡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. §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.

Radiolabelled rat fetal calvariae were used to determine if B16/F1 cells could degrade mineralised bone matrix. Consequently, ^{45}Ca-labelled calvariae were prepared and frozen/thawed to eliminate the possibility of interactions between tumour and bone cells. Devitalised calvariae were then exposed to 10^5 B16/F1 cells or their CM for 24 h.

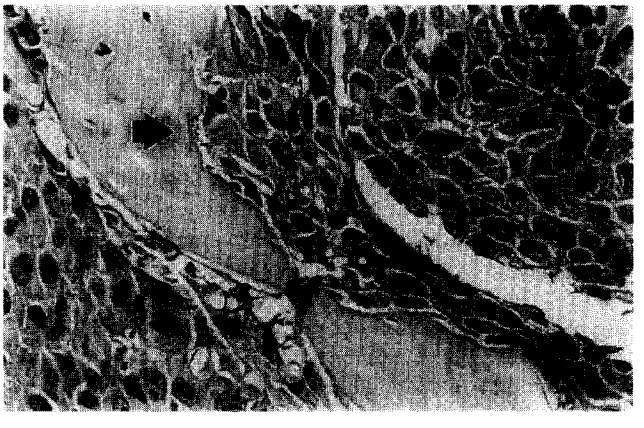

Figure 1. Experimental femoral bone metastasis by B16/F1 cells 14 days after intra-arterial injection of 10^5 cells. Melanoma cells are located directly over a partially-resorbed bone surface (arrow). Osteoclasts, osteoblasts or bone marrow cells are noticeably absent. ×250, haematoxylin and eosin.

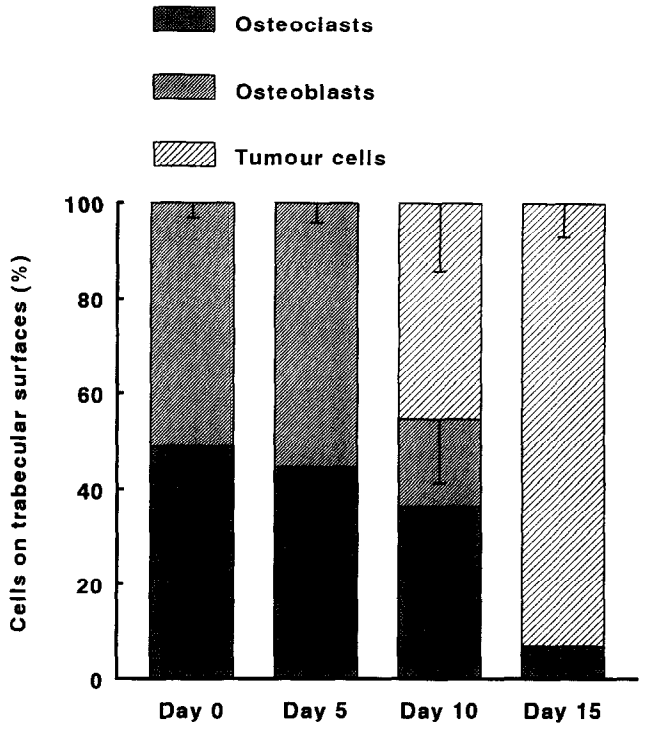

Figure 2. Proportion of bone and tumour cells in bone metastasis. Detection of TRAP activity was performed in plastic-embedded femurs on days 0, 5, 10 and 15 after intra-arterial injections of 10^5 murine B16/F1 melanoma cells. The number of osteoclasts was reduced in metastatic bones on days 10 and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

*Table 2. Degradation of Saos-2 and U-2 OS extracellular matrices by B16/F1 melanoma cells**

Conditions	Saos-2 matrix	U-2 OS matrix	Saos-2 matrix plus β-GPO_4 ‖
Unconditioned medium	99 ± 6	251 ± 24	780 ± 41
B16/F1-CM	123 ± 9†	313 ± 16	1199 ± 63
B16/F1 cells	115 ± 2†	614 ± 109‡	1511 ± 33
TPA-treated cells	144 ± 15†	859 ± 110§	1998 ± 88
CM from TPA-treated cells	–	–	1504 ± 39

*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 eight independent replicates and is expressed as disintegrations per minute. †$P < 0.05$ for test conditions versus unconditioned medium. ‡$P < 0.05$ for B16/F1 cells versus unconditioned and CM. §$P < 0.0001$ for TPA-treated cells versus unconditioned medium. ‖Partial mineralisation 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 versus unconditioned medium. $P < 0.005$ for B16/F1 cells versus CM. $P < 0.0001$ for B16/F1 cells treated with 400 ng/ml TPA versus untreated cells. $P < 0.05$ for CM from B16/F1 cells treated with 400 ng/ml TPA versus CM from untreated cells.

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 mineralised 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, 113–178 μm^2). Pits were not observed in bones incubated with medium alone (Figure 3a).

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

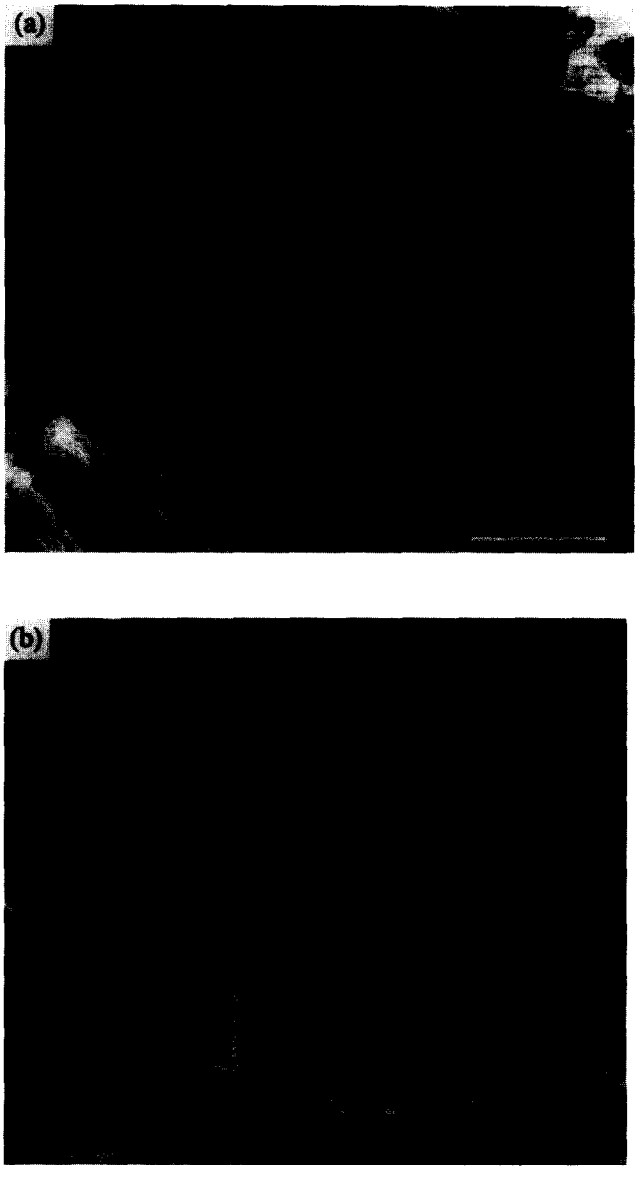

Figure 3. Resorption pits on bone surfaces exposed to B16/F1 melanoma cells. Scanning electron microscopy of the surface of longitudinal bovine cortical bone slices displayed depressions demarcated by a continuous rim and with a diameter of 12–15 µm (b). Pits were not observed in bones incubated with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

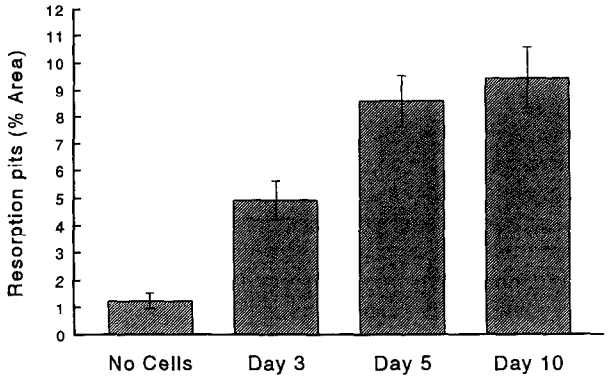

Figure 4. Presence of bone resorption pits on bovine bone slices exposed to B16/F1 melanoma cells. The per cent area of bone slices with resorption pits was determined by light microscopy according to Ritchie and associates [17]. The presence of tumour cells was associated with a time-related increase 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.

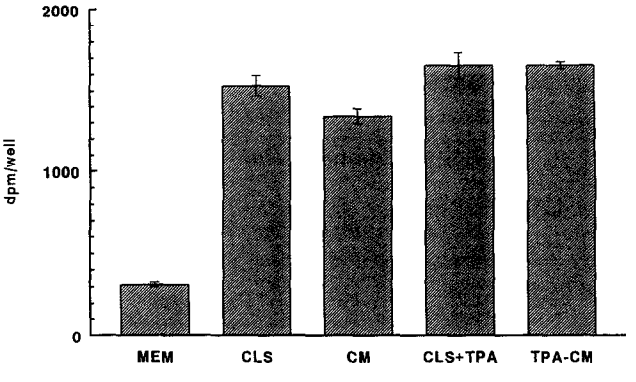

Figure 5. Degradation of type I collagen by B16/F1 cells. Conditioned medium or B16/F1 cells placed onto ^{3}H-labelled type I collagen induced radiolabel release ($P < 0.0001$ for test conditions versus unconditioned medium). Challenge of the tumour cells with 400 ng/ml TPA further increased collagenolysis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

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 catalysed 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 h 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. Gelatinolysis was observed at M_r 90000, 84000 and 64000 (Figure 6), and caseinolysis at M_r 54000 (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

Table 3. Effects of 1,10-phenanthroline on the degradation of U-2 OS matrix by conditioned medium from B16/F1 melanoma cells

Conditions*	^{3}H-proline release	Percentage inhibition	*P* value
Serum-free MEM	384 ± 14	–	–
B16/F1-CM	922 ± 31	–	Ref. category
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

*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 h at 37°C. Release of ^{3}H was then determined from medium aliquots. Each value represents the mean of eight independent replicates and is expressed as disintegrations per minute.

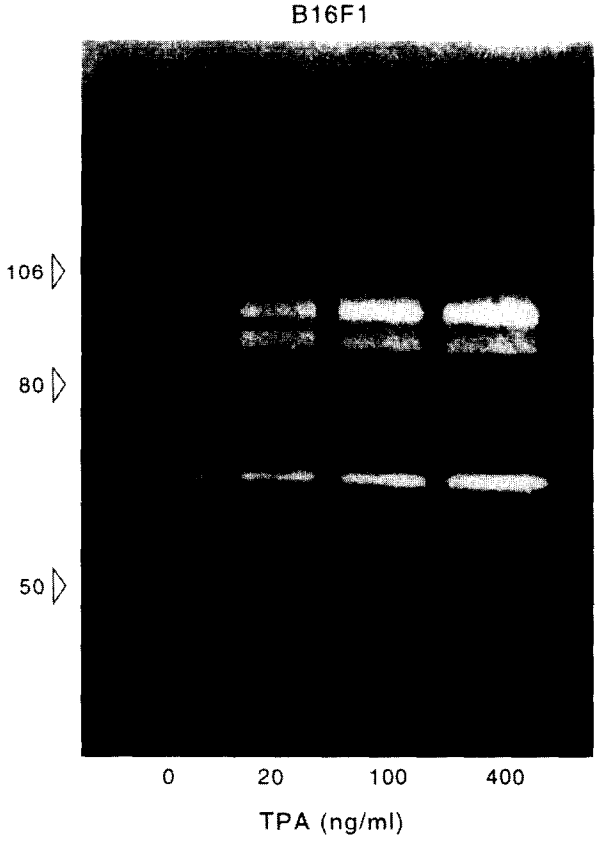

Figure 6. Gelatin enzymography of B16/F1-derived conditioned medium. Unstimulated B16/F1 cells displayed gelatinolytic activity at M_r 90000, 84000 and 64000. These activities were enhanced by exposure to increasing concentrations (0–400 ng/ml) of the phorbol ester TPA.

Table 4. Induction of matrix metalloprotenase release by treating B16/F1 cells with TPA

Molecular weight*	TPA 20 ng/ml†	TPA 100 ng/ml	TPA 400 ng/ml
90000 Da	7% (0–14)	16% (11–19)‡	19% (16–24)‡
84000 Da	5% (2–7)	10% (7–12)	9% (7–11)
64000 Da	1% (0–6)	5% (0–12)	6% (0–14)

*Cancer cell monolayers were incubated with serum-free medium in the presence of 20–400 ng/ml TPA for 24 h. Samples of B16/F1-CM were then collected. Protein-standardised 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 min. 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 h at 37 °C. Gels were then stained with 2.5% Coomassie Blue for 30 min, and destained with methanol/acetic acid/water (40:10:50). Densitometry of the gelatinolytic bands was performed on negative photographs of the gels. †Results represent percent increases over control (TPA 0 ng/ml), and are expressed as median (range) from six different experiments. ‡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.

1,10-phenanthroline (10 mM) for 48 h 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 h 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 M_r 52 000 caseinolytic band.

DISCUSSION

Bone metastases frequently exhibit osteolysis, a radiological indicator of their presence, and are associated with pain, fractures, hypercalcaemia 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 leukaemic cells can directly destroy bone [6]. In this study, we implicate metastatic melanoma cells and their matrix metalloproteinases as direct mediators of osteolysis.

Using histomorphometry and an animal model of experimental bone metastasis by murine melanoma cells, we 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 demonstrate that local osteolysis occurred in this animal model and suggests 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 tumour 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 mineralised matrices were 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 mineralised 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 has previously been 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 tumour cells *per se* did not appear to degrade bone directly. In particular, Boyde and associates [30] based this conclusion on unspecified ultrastructural features of resorption pits from a limited number of human spongy bone samples, in which tumour and bone cells were removed to identify the resorptive areas. Quinn and associates [28] addressed this question *in vitro* and showed that rat osteosarcoma cells were able to produce resorption pits in the presence of tumour-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 of active MMPs by melanoma cells. 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 antimouse antibodies. Since TPA is responsible for the induction of

many cellular pathways, the degradative induction observed may not necessarily be mediated only 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 phenomenon. Thus, we suggest that tumour-derived MMPs are mediators, at least in part, of direct osteolysis by murine melanoma cells.

The mechanisms by which cancer cells induce demineralisation of bone matrix are not known. Osteoclasts degrade bone by a combined action of membrane proton channels and proteinases, resulting in extracellular acidification, matrix demineralisation and degradation [31]. The presence of similar mechanisms on cancer cells has not been reported. We speculate that cancer cells may acidify their immediate micro-environment by other yet undefined mechanisms, in a less efficient manner than osteoclasts. Additionally, although cancer cells express type I collagen-binding integrins, it is unlikely that they will produce focalised degradation analogous to the $\alpha_v\beta_3$-mediated osteoclastic osteolysis, since osteoclasts have a complex machinery specialised in this task. Therefore, we propose that bone-metastasising cells, which secrete MMPs and induce matrix demineralisation, 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], tumour 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 MPPs are involved in tumour osteolysis is of importance since novel therapeutic approaches could be derived. Future characterisation 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 anti-osteoclastic agents do not completely prevent or abrogate metastasis-associated local osteolysis [35]. Since tumour 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.

1. Tubiana-Hulin M. Incidence, prevalence and distribution of bone metastases. *Bone* 1991, **12** (Suppl. 1), S9–S10.
2. Teitelbaum SL, Ross FP. Mechanisms of tumor-induced osteolysis. *Lab Invest* 1994, **71**, 453–455.
3. Garrett IR. Bone destruction in cancer. *Semin Oncol* 1993, **20**, 4–9.
4. Rosol TJ, Capen CC. Mechanisms of cancer-induced hypercalcemia. *Lab Invest* 1992, **67**, 680–702.
5. Athanasou NA, Quinn JMW. Human tumour-associated macrophages are capable of bone resorption. *Br J Cancer* 1992, **65**, 523–526.
6. Eilon G, Mundy GR. Direct resorption of bone by human breast cancer cells *in vitro*. *Nature* 1978, **276**, 726–728.
7. Mundy GR, Altman AJ, Gondek MD, Bandelin JG. Direct resorption of bone by human monocytes. *Science* 1977, **196**, 1109–1111.
8. Vaananen HK. Mechanism of bone turnover. *Ann Med* 1993, **25**, 353–359.
9. Aimes RT, Quigley JP. Matrix metalloproteinase-2 is an interstitial collagenase. *J Biol Chem* 1995, **270**, 5872–5876.
10. Fidler IJ. General considerations for studies of experimental cancer metastasis. *Methods Cancer Res* 1978, **15**, 399–439.
11. Arguello F, Baggs RB, Frantz CN. A murine model of experimental metastasis to bone and bone marrow. *Cancer Res* 1988, **48**, 6876–6881.
12. Merz WA, Schenk RK. Quantitative structural analysis of human cancellous bone. *Acta Anat* 1970, **75**, 54–66.
13. Gospodarowicz D, Lepine J, Massoglia S, Wood I. I. Comparison of the ability of basement membranes produced by corneal endothelial and mouse-derived endodermal PF-HR-9 cells to support the proliferation and differentiation of bovine kidney tubule epithelial cells *in vitro*. *J Cell Biol* 1984, **99**, 947–961.
14. Sakamoto S, Sakamoto M, Goldberg L, Colarusso L, Gotoh Y. Mineralization induced by beta-glycerophosphate in cultures leads to a marked increase in collagenase synthesis by mouse osteogenic MC3T3-E1 cells under subsequent stimulation with heparin. *Biochem Biophys Res Commun* 1989, **162**, 773–780.
15. Manishen WJ, Sivananthan K, Orr FW. Resorbing bone stimulates tumor cell growth. A role for the host microenvironment in bone metastasis. *Am J Pathol* 1986, **123**, 39–45.
16. Chambers TJ, Revell PA, Fuller K, Athanasou NA. Resorption of bone by isolated rabbit osteoclasts. *J Cell Sci* 1984, **66**, 383–399.
17. Ritchie CK, Maercklein PB, Fitzpatrick LA. Direct effect of calcium channel antagonists on osteoclast function: alterations in bone resorption and intracellular calcium concentrations. *Endocrinology* 1994, **135**, 996–1003.
18. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal Biochem* 1980, **102**, 196–202.
19. Rifas L, Fausto A, Scott MJ, Avioli LV, Welgus HG. Expression of metalloproteinases and tissue inhibitors of metalloproteinases in human osteoblast-like cells: differentiation is associated with repression of metalloproteinase biosynthesis. *Endocrinology* 1994, **134**, 213–221.
20. Ossowski L. Invasion of connective tissue by human carcinoma cell lines: requirement for urokinase, urokinase receptor, and interstitial collagenase. *Cancer Res* 1992, **52**, 6754–6760.
21. Knauper V, Lopez-Otin C, Smith B, Knight G, Murphy G. Biochemical characterization of human collagenase-3. *J Biol Chem* 1996, **271**, 1544–1550.
22. Woessner JF Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 1991, **5**, 2145–2154.
23. Roodman GD. Role of cytokines in the regulation of bone resorption. *Calcif Tissue Int* 1993, **53** (Suppl. 1), S94–S98.
24. Francini G, Petrioli R, Maioli E, *et al.* Hypercalcemia in breast cancer. *Clin Exp Metast* 1993, **11**, 359–367.

25. Galasko CSB. Mechanisms of lytic and blastic metastatic disease of bone. *Clin Orthop Relat Res* 1982, **169**, 20–27.
26. Quinn JMW, Athanasou NA. Tumour infiltrating macrophages are capable of bone resorption. *J Cell Sci* 1992, **101**, 681–686.
27. Eilon G, Mundy GR. Association of increased cyclic adenosine 3′:5′-monophosphate content in cultured human breast cancer cells and release of hydrolytic enzymes and bone-resorbing activity. *Cancer Res* 1983, **43**, 5792–5794.
28. Quinn JMW, Matsumara Y, Tarin D, McGee JO, Athanasou NA. Cellular and hormonal mechanisms associated with malignant bone resorption. *Lab Invest* 1994, **71**, 465–471.
29. Canalis E, McCarthy TL, Centrella M. Effects of desamino-(1,3)-insulin-like growth factor I on bone cell function in rat calvarial cultures. *Endocrinology* 1991, **129**, 534–541.
30. Boyde A, Maconnachie E, Reid SA, Delling G, Mundy GR. Scanning electron microscopy in bone pathology: review of methods, potential and applications. *Scanning Electron Microsc* 1986, 4, 1537–1554.
31. Everts V, Delaisse J-M, Korper W, Niehof A, Vaes G, Beertsen W. Degradation of collagen in the bone-resorbing compartment underlying the osteoclast involves both cysteine-proteinases and matrix metalloproteinases. *J Cell Physiol* 1992, **150**, 221–231.
32. Teitelbaum SL. Bone remodeling and the osteoclast. *J Bone Miner Res* 1993, **8** (Suppl. 2), S523–S525.
33. Galasko CSB, Bennett A. Relationship of bone destruction in skeletal metastases to osteoclast activation and prostaglandins. *Nature* 1976, **263**, 508–510.
34. Galasko CSB. Mechanisms of bone destruction in the development of skeletal metastases. *Nature* 1976, **263**, 507–508.
35. Mundy GR. Mechanisms of osteolytic bone destruction. *Bone* 1991, **12** (Suppl. 1), S1–S6.

Acknowledgements—This study was supported by the Medical Research Council of Canada, Operating Grant MT-10409, to F.W.O. and G.S. O.H.S.-S. was supported in part by an Ontario Graduate Scholarship from the Ministry of Colleges and Universities of the Province of Ontario.