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Research Article

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Inhibition of Osteolytic Bone Metastasis of Breast Cancer by Combined Treatment with the Bisphosphonate Ibandronate and Tissue Inhibitor of the Matrix Metalloproteinase-2

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Abstract

Multiple steps are involved in the metastasis of cancer cells from primary sites to distant organs. These steps should be considered in the design of pharmacologic approaches to prevent or inhibit the metastatic process. In the present study, we have compared the effects of inhibiting several steps involved in the bone metastatic process individually with inhibition of both together. The steps we chose were matrix metalloproteinase (MMP) secretion, likely involved in tumor cell invasion, and osteoclastic bone resorption, the final step in the process. We used an experimental model in which inoculation of human estrogen-independent breast cancer MDA-231 cells into the left cardiac ventricle of female nude mice causes osteolytic lesions in bone. To inhibit cancer invasiveness, the tissue inhibitor of the MMP-2 (TIMP-2), which is a natural inhibitor of MMPs, was overexpressed in MDA-231 cells. To inhibit bone resorption, a potent bisphosphonate, ibandronate (4 μg/mouse) was daily administered subcutaneously. Nude mice received either; (*a*) nontransfected MDA-231 cells; (*b*) nontransfected MDA-231 cells and ibandronate; (*c*) TIMP-2–transfected MDA-231 cells; or (*d*) TIMP-2–transfected MDA-231 cells and ibandronate. In mice from group *a*, radiographs revealed multiple osteolytic lesions. However, in mice from group *b* or group *c*, osteolytic lesions were markedly decreased. Of particular note, in animals from group *d* receiving both ibandronate and TIMP-2–transfected MDA-231 cells, there were no radiologically detectable osteolytic lesions. Survival rate was increased in mice of groups *c* and *d*. There was no difference in local enlargement in the mammary fat pad between nontransfected and TIMP-2–transfected MDA-231 cells. These results suggest that inhibition of both MMPs and osteoclastic bone resorption are more efficacious treatment for prevention of osteolytic lesions than either alone, and suggest that when therapies are designed based on the uniqueness of the bone microenvironment and combined with several common steps in the metastatic process, osteolytic bone metastases can be more efficiently and selectively inhibited. (*J. Clin. Invest.* 1997. 99:2509–2517.) Key words: breast cancer • bone metastasis • ibandronate • TIMP-2 • osteoclast

Introduction

Cancer dissemination to distant organs consists of multiple sequential steps which include diverse and complex cellular and molecular events (1, 2). In most cases, cancer shows a selective nonrandom pattern of metastasis to particular organs, depending on the site where the primary tumor occurs (3, 4). Breast cancer is known to have a strong predilection for spreading to bone (5–8). Breast cancer metastasis to bone can broadly be divided into two major steps, i.e., the general steps which occur before breast cancer reaches bone and, are common to cancer metastasis to any organ, and specific steps which occur during breast cancer colonization in bone (8). In both general and specific steps, a variety of molecules including cell adhesion molecules (CAMs),[1] matrix metalloproteinases (MMPs), growth factors, and cytokines have been implicated (9). Osteoclasts, which are the unique multinucleated giant cells responsible for bone destruction, are essential for the development and progression of osteolytic bone metastases. We have previously demonstrated that expression of the cell-to-cell adhesion molecule E-cadherin gene (10) in a human estrogen-independent breast cancer cell line MDA-MB-231 (MDA-231) decreases bone metastasis (11). Using the same experimental metastasis model, it was also found that a neutralizing monoclonal antibody to parathyroid hormone-related protein (PTH-rP), which is a potent stimulator of osteoclastic bone resorption, suppressed bone metastases (12). In addition, we have reported that the bisphosphonate risedronate, a specific inhibitor of osteoclastic bone resorption, inhibited osteolytic bone metastases and decreased tumor bulk in bone in vivo (13). These results indicate that these molecules and osteoclasts are the

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1. *Abbreviations used in this paper:* BMSEC, bone marrow sinus-derived endothelial cell; CAMs, cell adhesion molecules; CM, conditioned medium; ECM, extracellular matrix; MDA-231, MDA-MB-231; MDA-231. EV, MDA-231 cells transfected with empty vector; MDA-231.P, nontransfected MDA-231 cells; MDA-231.TIMP-2, MDA-231 cells overexpressing TIMP-2; αMEM, alpha minimal essential medium; MMPs, matrix metalloproteinases; PTH-rP, parathyroid hormone-related protein; RT, room temperature; TIMP-2, tissue inhibitor of the matrix metalloproteinase-2.

potential targets in the design of pharmacological agents to inhibit breast cancer metastasis to bone. An involvement of both general and specific steps in bone metastasis suggests that simultaneous inhibition of these two distinct steps seems likely to provide a more effective approach than does inhibition of either step alone.

In the present study, we first determined the role of MMPs, which have been implicated in cancer invasiveness (9), in osteolytic bone metastases formed by MDA-231 cells in an experimental metastasis model in nude mice. We studied this by overexpressing tissue inhibitor of the matrix metalloproteinase-2 (TIMP-2), a natural inhibitor of MMPs, into MDA-231 cells. We then examined the effects of a combination of TIMP-2 overexpression and ibandronate, another potent bisphosphonate (14), compared with those of each single treatment. MDA-231 cells overexpressing TIMP-2 (MDA-231.TIMP-2 cells) showed decreased osteolytic bone metastases. Ibandronate suppressed the progression of established osteolytic bone metastases and the development of new osteolytic bone metastases by nontransfected MDA-231 cells (MDA-231.P) cells. When ibandronate was tested in animals that were inoculated with MDA-231.TIMP-2 cells, there was an even more dramatic decrease in osteolytic bone metastases. Our results demonstrate that MMPs play an important role in breast cancer metastasis to bone. They also suggest that a combination of bone-specific and common metastasis inhibitors is much more effective in inhibiting osteolytic bone metastases than single treatment alone.

Methods

MDA-231:a human breast cancer cell line. The estrogen-independent human breast cancer cell line MDA-231 (15) was cultured in DMEM (Hazleton Biologics, Inc., Lenexa, KS) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT) and 1% penicillin-streptomycin solution (Life Technologies, Inc., Grand Island, NY) in a humidified atmosphere of 5% CO_2 in air.

Transfection. Human MI/TIMP-2 cDNA was transfected into MDA-231 cells using a calcium phosphate precipitation technique according to the methods described (16). For selection of transfected colonies, the hygromycin B phosphotransferase gene was cotransfected. Colonies resistant to 0.6 mg/ml hygromycin B (Sigma Chemical Co., St. Louis, MO) were picked up and clones were established by limiting dilution and determined for the secretion of TIMP-2 by immunoblotting and gelatinase assay (see below). Control MDA-231 cells received pcNDAI (Invitrogen, San Diego, CA) empty vector (MDA-231.EV).

Intracardiac injections of MDA-231 in nude mice. Intracardiac injection of MDA-231 was performed according to the technique described previously (11, 13). Subconfluent MDA-231 cells were fed with fresh medium 24 h before intracardiac injections. Cells (1×10^5) were suspended in 0.1 ml of PBS and injected into the left heart ventricle of 4-wk-old, female BALB/c-nu/nu mice (Harlan Industries, Houston, TX) with 27-gauge needle under the anesthesia with pentobarbital (0.05 mg/gram). Animals were kept in our animal facilities for 4 to 7 wk as described (17). Weight of animals and excised tumors was determined by using a digitalized weigher (Lum-O-Gram™; Ohaus Scale Corporation, Florham Park, NJ).

Treatment schedules. The bisphosphonate ibandronate (Boehringer Mannheim GmbH, Mannheim, Germany) was diluted in PBS and the pH was adjusted to 7.4. The dose given is expressed as free acid equivalents of the sodium salt monohydrate. The drug was administered daily by subcutaneous injection in different treatment schedules as depicted (see Fig. 6). Control animals received injections of vehicle.

Determination of number and area of bone metastases. The number of osteolytic bone metastases was enumerated on radiographs as described (11, 13, 18). Animals were anesthetized deeply, laid down in prone and lateral positions against the films (22 × 27 cm; X-OMAT AR; Eastman Kodak Co., Rochester, NY), and exposed to an x ray at 35 kV for 6 s using a Faxtron radiographic inspection unit (43855A; Faxitron X-ray Corporation, Buffalo Grove, IL). Films were developed using a RP X-OMAT processor (M6b; Eastman Kodak Co.). All of the radiographs of bones in nude mice were evaluated extensively and carefully by three different individuals including one radiologist who were without knowledge of the experimental protocols. On radiographs, the number and area of osteolytic metastatic foci as small as 0.5 mm in diameter, which were recognized as demarcated radiolucent lesions in the bone, were quantitatively assessed using a computer-assisted JAVA image analysis system (Jandel Scientific, Corte Madera, CA).

Histological examination of metastatic cancer burden. The details of these methods were described previously (13). In brief, forelimbs and hindlimbs from animals in each treatment group were fixed with 10% formalin in PBS (pH 7.2) and decalcified in 14% EDTA solution for 2–3 wk. Paraffin sections were made following conventional methods. The area of metastatic cancer infiltrations was measured in the distal femoral and proximal tibial metaphyses of both limbs in longitudinal decalcified sections stained by hematoxylin and eosin.

Immunoblotting. Cells were lysed in lysis buffer containing 20 mM Tris pH 8.0, 2 mM $CaCl_2$, 150 mM NaCl, 1% NP-40, 0.1% SDS, and protease inhibitors (20 mM leupeptin, 1 mM phenylmethylsulfonylfluoride, 1% aprotinin) at 4°C, centrifuged at 10,000 *g* for 10 min at 4°C, and determined for protein amounts using Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). The lysates were boiled for 5 min, separated on 12% SDS-PAGE, and transferred onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) in transblotting buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol (pH 8.0). The membranes were incubated with blocking buffer consisting of 5% nonfat dry milk in 50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$ for 2 h and then with polyclonal antibodies to recombinant MI/TIMP-2 (15) diluted 1:200 in the same buffer for 1 h, washed 5 times with TBST, incubated with HRP-conjugated protein A (Capel, Durham, NC) (diluted 1:2000 in TBST containing 5% nonfat dry milk) for 1 h. After the membranes were washed five times with TBST, then two times with TBS, the signal was visualized with ECL detection system (DuPont NEN, Boston, MA).

Colony formation assay. Anchorage-independent growth of MDA-231 was determined as previously described (19). 1 ml of agarose (Sea-Plaque; FMC Corp. Bioproducts, Rockland, ME) at 0.4% (wt/vol) in DMEM supplemented with 10% FBS containing 300 cells was overlaid onto a 1-ml bottom layer of 0.6% agarose in the same culture medium in 35-mm tissue culture plates with grid at the bottom (Sarstedt, Newton, NC). Plates were incubated for 14 d in a humidified CO_2 incubator at 37°C and colonies > 100 μm in diameter were manually counted under an inverted microscope.

Tumorigenicity of MDA-231 cells in nude mice. Cells (2×10^6) were suspended in 0.2 ml of the mixture (1:1) of PBS and Matrigel® (Collaborative Research, Bedford, MA) and inoculated into the right thoracic mammary fat pad of female nude mice using 23 gauze needles as previously described (20). 4 wk later, tumors formed were excised and determined for their weight.

Gelatin zymography and reverse zymography. Serum-free CM of confluent MDA-231 cells which were cultured on extracellular matrix (ECM)-coated plates were concentrated 50-fold on Centricon-10 (10 kD cut-off; Amicon Inc., Beverly, MA), determined for protein amounts using Bio-Rad DC protein assay kit and stored at −70°C until assay. MMP and TIMP-2 activity in the media was assessed by gelatin zymography and reverse zymography, respectively, following the methods described previously (16). In brief, the concentrated media (20 μg/lane) were run on SDS-PAGE (7.5% for zymography and 12% for reverse zymography containing 1 mg/ml gelatin) for 3 h at 15 mA at 4°C. The gels were incubated in Triton X-100 (2.5%, vol/vol)

for 1 h, then for reverse zymography with gelatinase overnight at 37°C and for zymography in 50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, 0.02% Brij 35 pH 7.6 overnight at 37°C. The gels were then stained with 0.5% (wt/vol) Coomassie brilliant blue and destained in methanol/acetic acid/water (50:10:40). White bands indicate gelatinase activity and a dark band indicates TIMP-2 activity.

Gelatinase activity. To further confirm the efficacy of TIMP-2 cDNA transfection into MDA-231 cells, gelatinase activity was determined using [^{3}H]gelatin which was prepared by heat denaturation of [^{3}H]collagen (New England Nuclear) for 30 min, 60°C in 0.1 M phosphate buffer, pH 7.5, containing 0.2M NaCl as described (21). [^{3}H]gelatin (0.2 mg/ml, 2.4 μCi/mg) was incubated with 20 μg concentrated MDA-231 CM in 50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35, pH 7.5 in a final volume of 220 μl. The incubation was run for 20 min at 37°C and terminated by the addition of EDTA/BSA solution (final concentration 2.5 mM/0.03%). Undigested [^{3}H] gelatin was precipitated with 0.05% tannic acid/2% trichloroacetic acid and the radioactivity of the supernatants was measured in a liquid scintillation counter (22, 23).

Transendothelial invasion assay. Assay was carried out based on the method previously described (24) using murine bone marrow sinus-derived endothelial cell line (BMSEC; generously provided by Dr. Hawley, University of Toronto, Toronto, Canada) (25) and Biocoat Matrigel® invasion chambers (24-well plates; Becton Dickinson & Co., Bedford, MA) with modifications as suggested by the manufacturer. BMSEC (3×10^5) were plated onto Matrigel-coated PET membrane (pore size 8 μm) in the cell culture insert, cultured until they reached confluency and treated with interleukin 1β (2 ng/ml) for 4 h. MDA-231 cells (1.5×10^4) were then seeded on the BMSEC and incubated for 15 h in a CO_2 incubator. At the end of incubation, cells on the upper surface of the membrane were completely removed off by cotton swab and cells on the lower surface of the membrane were fixed with 40% formalin and stained with 0.1% crystal violet. Stained cells were counted randomly in 10 high-power fields under the microscope at 100 magnification.

ECM-coated plates. Bone ECM-coated plates were prepared as described (26). Human osteoblastic osteosarcoma MG-63 cells were cultured in alpha minimum essential medium (αMEM) supplemented with 5% FBS and 100 mM ascorbic acid for 10–14 d, rinsed with PBS, and treated with 0.5% Triton for 10 min room temperature (RT). Triton-solubilized fractions were gently aspirated and the remaining fractions on the bottom surface of culture plates were washed with PBS three times, treated with 25 mM NH_4OH for 10 min at RT, and rinsed with PBS at 4°C. Bone ECM-coated plates were stored in PBS at 4°C. Tissue culture plates coated with other types of ECMs such as matrigel, fibronectin, laminin, type I collagen and poly-L-lysine were purchased from Becton Dickinson Labware.

Statistical analysis. All data were analyzed by Mann-Whitney test for nonparametric samples. The statistical difference of survival rate of the animals was analyzed by generalized Wilcoxon test. All data were presented as the mean±SEM.

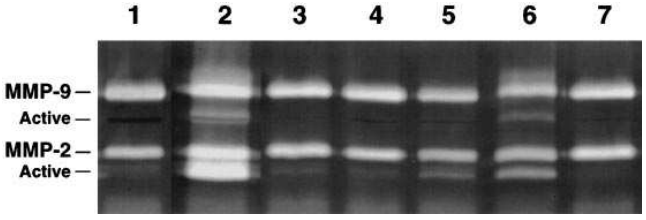

Figure 1. Gelatin zymogram of MDA-231 CM. Serum-free CM of confluent MDA-231 cells grown on plastic (lane *1*), bone ECM (lane *2*), laminin (lane *3*), fibronectin (lane *4*), type I collagen (lane *5*), matrigel (lane *6*) and poly-L-lysine (lane *7*) was harvested, concentrated, and subjected to gelatin zymography. MMP-9, 92-kD gelatinase (gelatinase B); MMP-2, 72-kD gelatinase (gelatinase A).

Results

MMP production by MDA-231 cells. We first examined whether MDA-231 cells produced MMPs by gelatin zymography. MDA-231 cells which were grown on plastic tissue culture dishes secreted latent forms of 92-kD gelatinase (MMP-9 or gelatinase B) and 72-kD gelatinase (MMP-2 or gelatinase A) (Fig. 1, lane *1*). In contrast, when MDA-231 cells were cultured on bone ECM-coated dishes, the secretion of the active form was markedly induced (Fig. 1, lane *2*). Significant levels of the active forms of MMP-9 and MMP-2 were also detected when MDA-231 cells were cultured on matrigel-coated plates (Fig. 1, lane *6*), whereas no production of the active forms was observed in MDA-231 cells which were grown on laminin, fi-

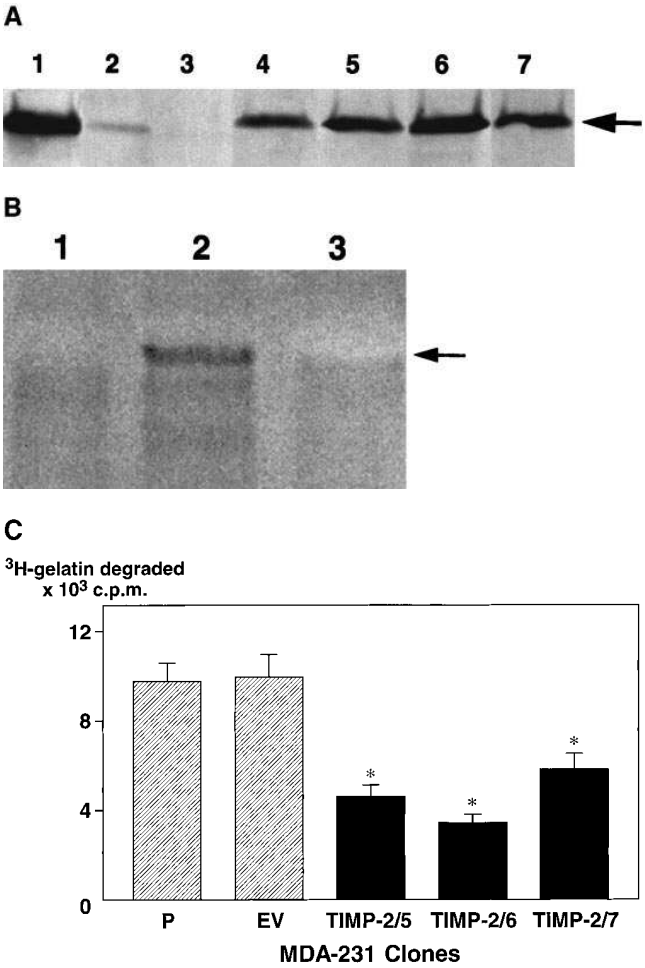

Figure 2. (*A*) Western blotting of TIMP-2 expression in MDA-231.P (lane *2*), MDA-231.EV (lane *3*), or MDA-231.TIMP-2 clones (lanes *4–7*). CM of MDA-231 transfectants was harvested, concentrated, and determined for TIMP-2 expression by Western blotting. Lane *1*, positive control, c-Ha-ras-1–transfected rat embryo cell CM (reference 16). Transfectants on lane *5*, *6*, and *7* were designated as MDA-231.TIMP-2/5, TIMP-2/6, and TIMP-2/7, respectively, and used in the following experiments. (*B*) Reverse zymogram. Lane *1*, MDA-231.P; lane *2*, MDA-231.TIMP-2/6; lane *3*, MDA-231.EV. (*C*) Gelatinase activity of MDA-231 clones. All clones were grown on bone ECM to activate progelatinases. Data shown are mean±SEM ($n = 4$). *Significantly different from MDA-231.P or MDA-231.EV ($P < 0.01$).

bronectin, and poly-L-lysine (Fig. 1, lanes *3*, *4*, and *7*). On type 1 collagen, there was a marginal increase in active form of MMP-2 (Fig. 1, lane *5*).

Transfection of TIMP-2 cDNA into MDA-231 cells. Subsequent to transfection, hygromycin selection and cloning by limiting dilution, several subclones of MDA-231 cells which overexpressed TIMP-2 were obtained. Using a rabbit polyclonal antiserum raised against recombinant TIMP-2 (16), we determined the expression of TIMP-2 by Western blotting (Fig. 2 *A*). MDA-231.P cells and MDA-231.EV cells exhibited marginal levels of TIMP-2 expression (Fig. 2 *A*, lanes *2* and *3*, respectively). In contrast, several clones of MDA-231.TIMP-2 showed increased TIMP-2 expression (Fig. 2 *A*, lanes *4*–*7*). In the following experiments, we used three clones of MDA-231.TIMP-2, i.e., clones in Fig. 2, lanes *5*–*7*. These clones were designated as MDA-231.TIMP-2/5, MDA-231.TIMP-2/6, and MDA-231.TIMP-2/7, respectively.

Consistent with the result of Western blotting, MDA-231.TIMP-2/6 showed TIMP activity on reverse zymogram (Fig. 2 *B*) and gelatinase activity in MDA-231.TIMP subclones was markedly decreased compared with MDA-231.P or MDA-231.EV (Fig. 2 *C*). The results demonstrate that TIMP-2 cDNA transfected into MDA-231 cells is functionally active and inhibits gelatinase activity.

Because it has been reported that TIMP-2 gene transfection causes a change in cell shape and adhesion to ECMs (27), we examined whether this was the case for these MDA-231.TIMP-2 subclones. There were no changes in cell shape and attachment to laminin, type I and IV collagen and fibronectin in MDA-231.TIMP-2 subclones compared with MDA.231.P or MDA-231.EV (data not shown).

Growth of MDA-231 transfectants. Before we determined the capacity of these transfected clones to form osteolytic bone metastases, proliferation of these clones in vitro and in vivo was examined, because TIMP-2 has been found to either stimulate (28) or inhibit (29, 30) cell growth. There was no difference in the growth of MDA-231.TIMP-2 subclones in monolayer (data not shown). Likewise, anchorage-independent growth of each MDA-231.TIMP-2 subclone in soft agar was not altered compared with MDA-231.P or MDA-231.EV (Fig. 3 *A*). Furthermore, tumor formation in the mammary fat pad (orthotopic site) in female nude mice was not changed, either (Fig. 3 *B*). These data demonstrated that transfection of TIMP-2 cDNA had no effects on the growth and tumorigenicity of MDA-231 cells.

Transendothelial invasiveness of MDA-231.TIMP-2 clones. Since it has been realized that cancer cells are required to actively penetrate through the endothelium of the bone marrow sinus to migrate into the marrow cavity (31), we determined the ability of MDA-231 cells to pass through the BMSEC using the transendothelial invasion assay. MDA-231.TIMP-2/6 displayed decreased invasiveness compared with MDA-231.P or MDA-231.EV (Fig. 4).

Formation of osteolytic bone metastases by MDA-231.TIMP-2 clones. We then examined these subclones for the capacity of forming osteolytic bone metastases by inoculating into the left cardiac ventricle in female nude mice. We have demonstrated on numbers of occasions that using this technique, MDA-231 cells selectively colonize bone and form osteolytic bone metastases but very rarely spread to nonbone or-

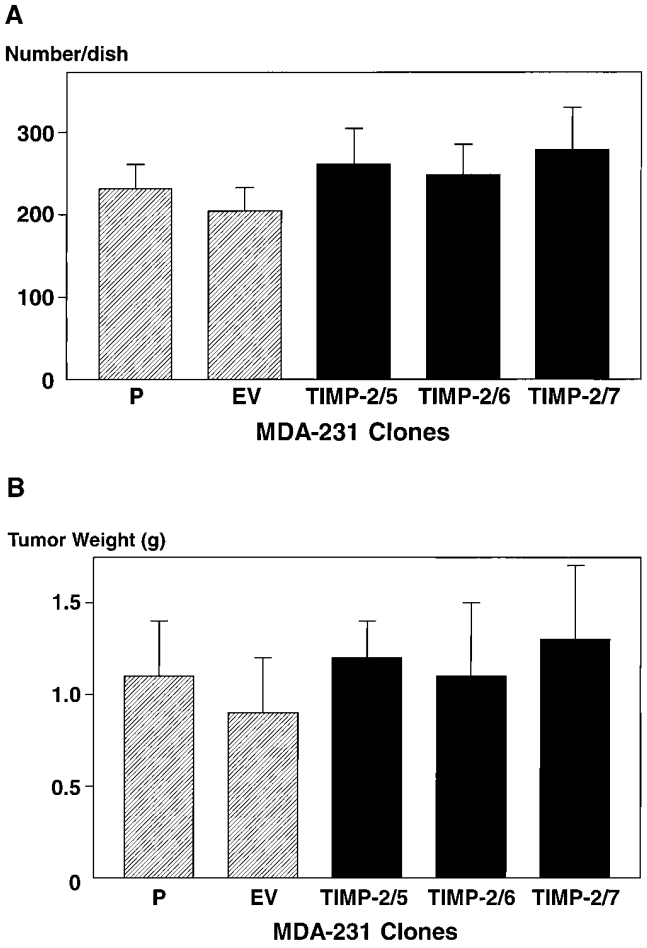

Figure 3. (*A*) Growth of MDA-231 transfectants in soft agar. 500 cells/24-well were inoculated and grown for 14 d. Colonies > 100 μm in diameter were manually counted under microscope. Data shown are mean±SEM ($n = 4$). (*B*) Tumor formation of MDA-231 transfectants in the mammary fat pad in nude mice. Two million cells in the mixture of PBS and matrigel (1:1) were inoculated. 4 wk later, tumors formed were excised and weighed. Data shown are mean±SEM ($n =$ 6 animals/group).

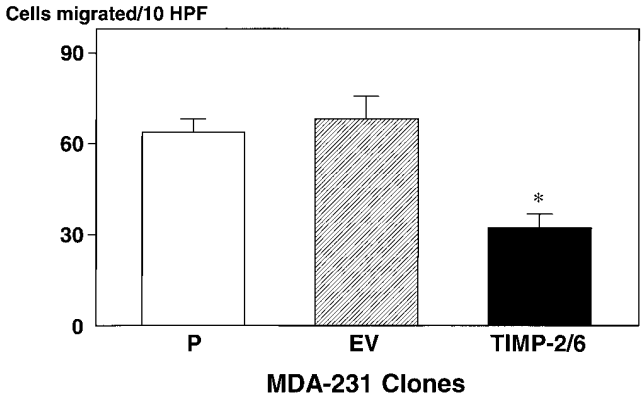

Figure 4. Invasiveness of MDA-231.TIMP-2/6 through the BMSEC in transendothelial cancer invasion assay. Data shown are mean±SEM ($n = 4 \times 2$ experiments = 8). *Significantly different from MDA-231.P or MDA-231.EV ($P < 0.01$).

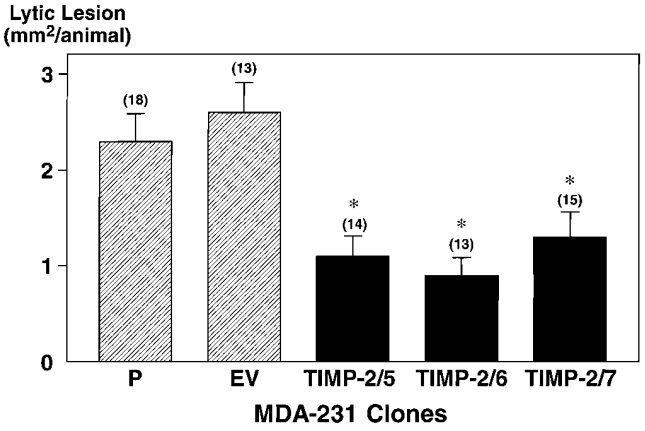

Figure 5. Formation of osteolytic bone metastases by MDA-231.TIMP-2 cells. MDA-231.P, MDA-231.EV, or MDA-231.TIMP-2/5, TIMP-2/6, and TIMP-2/7) cells (1×10^5/animal) were inoculated into the left cardiac ventricle of 4-wk-old female nude mice. 4 wk later, osteolytic lesions in the distal femur and proximal tibiae of right and left legs were determined for their area on radiographs by computer-assisted image analyser. Numbers in parentheses are numbers of animals studied. Data shown are mean±SEM. *Significantly smaller than control ($P < 0.01$).

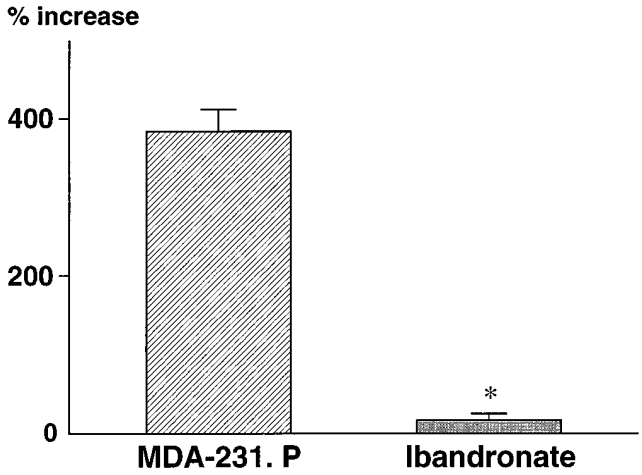

Figure 7. Effect of ibandronate on the progression of established osteolytic bone metastases. Ibandronate (4 μg/animal, SC) was given daily to animals with established osteolytic bone metastases from day 17 to 28 (see Fig. 6 *A*). Percent increase refers to mm²/animal and was calculated as: area at day 28 − area at day 17/area at day 17 × 100. Data shown are mean±SEM ($n = 8$). *Significantly different from untreated group ($P < 0.01$).

gans including lung, liver, spleen, kidney, and brains (11, 13). As shown in Fig. 5, MDA-231.TIMP-2/5, MDA-231.TIMP-2/6, and MDA-231.TIMP-2/7 manifested significantly decreased osteolytic bone metastases compared with MDA-231.P or MDA-231.EV. The radiographs also displayed that TIMP-2 cDNA transfection into MDA-231 cells caused a reduction in size of osteolytic lesion (data not shown).

Effects of ibandronate on osteolytic bone metastases of MDA-231.P cells. The effects of the specific inhibitor of osteoclastic bone resorption, ibandronate, were tested on (*a*) the progression of the established osteolytic bone metastases. This experiment represents the clinical situation in which ibandronate is given to breast cancer patients with detectable osteolytic bone metastases and (*b*) the formation of new osteolytic bone metastases. This experiment represents the clinical situation in which ibandronate is given to breast cancer patients in a preventive manner. In both experiments, MDA-231.P cells were used. The protocol of these experiments were depicted in Fig. 6, *A* and *B*, respectively.

Ibandronate (4 μg/animal, subcutaneously [s.c.]) administered daily to animals with established osteolytic bone metastases from day 17 to day 28 (Fig. 6 *A*) significantly suppressed an increase in size of osteolytic lesions determined radiologically (Fig. 7).

In the second experiments, ibandronate (4 μg/animal, s.c.) given daily for 3 wk prior to MDA-231.P cell inoculation (Fig. 6 *B*) markedly prevented the formation of new osteolytic bone metastases (Fig. 8).

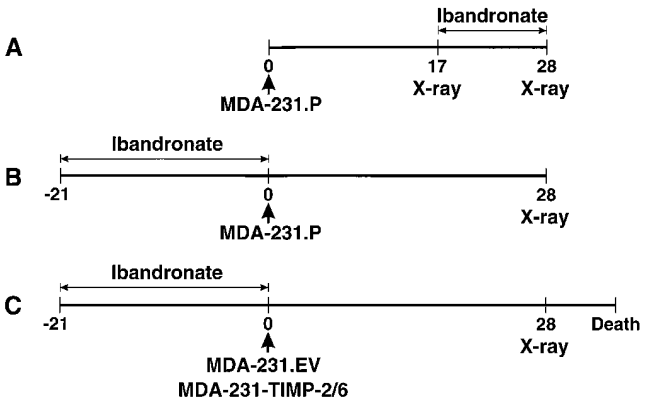

Figure 6. Experimental protocol of ibandronate administration. Note that in *A* and *B*, MDA- 231.P cells were used and in *C*, MDA-231.TIMP-2/6 cells were used. In *C*, survival was also determined.

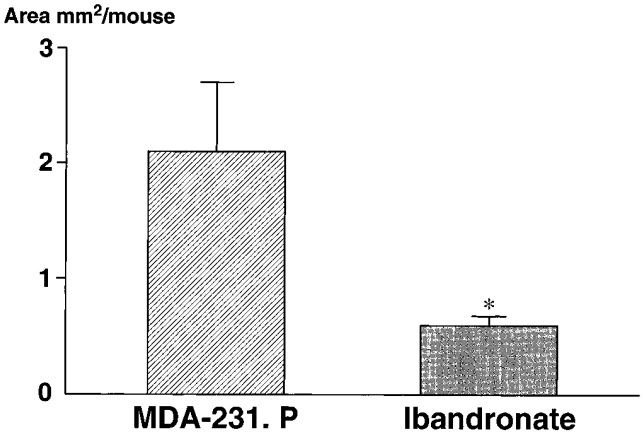

Figure 8. Effect of ibandronate on the development of new osteolytic bone metastases. Ibandronate (4 μg/animal, SC) was given daily for 3 wk before MDA-231.P cell inoculation into the heart of animals (see Fig. 6 *B*). Quantitation of lytic lesions. Data shown are mean±SEM ($n = 13$, combination of two separate experiments). *Significantly different from untreated groups ($P < 0.005$).

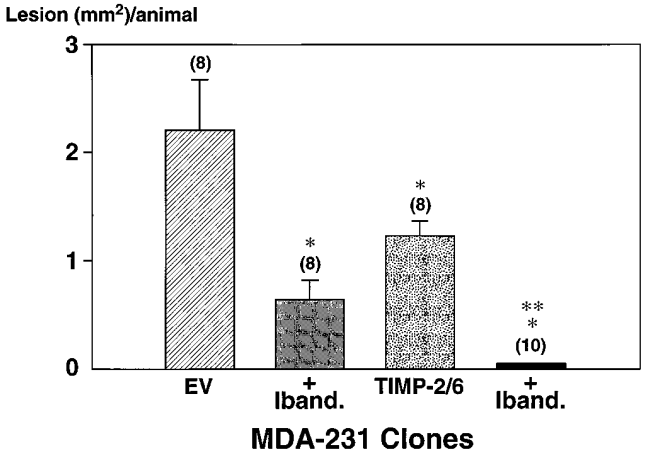

Figure 9. Effect of a combination of ibandronate and TIMP-2 overexpression on the development of new osteolytic bone metastases. Some groups of mice were given ibandronate (4 μg/animal, SC) and other groups of mice given PBS for 3 wk before cell inoculation. MDA-231.EV or MDA-231.TIMP-2/6 cells (1×10^5/animal) were inoculated into the left cardiac ventricle of female nude mice of each group. 4 wk later, osteolytic lesions were radiologically detected and quantitated for their area using image analyser (see Fig. 6 *C*). Data shown are mean±SEM. Numbers in parentheses are numbers of animals studied. *Significantly different from empty vector (*EV*). **Significantly different from ibandronate or TIMP-2 alone ($P < 0.01$).

Effects of a combination of ibandronate and TIMP-2 overexpression on established osteolytic bone metastases. We next determined the combined effects of ibandronate and TIMP-2 overexpression on the development of osteolytic bone metastases (Fig. 6 *C*). As demonstrated in Fig. 9, a combination of ibandronate and TIMP-2 dramatically suppressed the development of osteolytic bone metastases compared with ibandronate administration or MDA-231.TIMP-2/6 inoculation alone.

Histological examination of bones with osteolytic bone metastases. Histological examination revealed that metastatic tumor burden and osteoclastic bone destruction were profoundly decreased in the bone of animals which received both MDA-231.TIMP-2/6 and ibandronate compared with bones of animals which received each treatment alone (Fig. 10).

Effects of a combination of ibandronate and TIMP-2 overexpression on survival of animals with established bone metastases. Using the same experimental protocol as Fig. 9 (Fig. 6 *C*), we determined the effects of each or combination of these treatments on the survival of animals. Ibandronate alone did not prolong the survival of animals (Fig. 11). In contrast, TIMP-2 overexpression caused a marked increase in the lifespan of animals (Fig. 11). Ibandronate did not further prolong the survival of animals carrying MDA-231.TIMP-2/6 cells.

Discussion

Cancer spread to a particular secondary site is achieved only when cancer cells successfully complete a series of multistep processes. Inhibition of these steps by selective pharmacological agents should, thus, lead to the suppression of cancer metastasis to that site. In the present study, we attempted to inhibit breast cancer metastasis to bone, because breast cancer is the most frequently occurring female cancer in the United States (32), bone is the most common site of breast cancer colonization (6, 8) and there are no satisfactory pharmacological treatments for breast cancer cells colonized in bone at the present time. We demonstrated that overexpression of TIMP-2, which is a natural inhibitor of MMPs which are also known to play a crucial role in several common steps of cancer metastasis (33) into a human estrogen-independent breast cancer cell line MDA-231, decreased osteolytic bone lesions and increased survival rate in tumor-bearing nude mice. Moreover, we also showed that a newly developed potent bisphosphonate, ibandronate, reduced osteolytic bone lesions, confirming our previous data obtained using another potent bisphosphonate, risedronate (13). Since these bisphosphonates are specific inhibitors of osteoclastic bone resorption, these results suggest that pharmacological manipulation of the bone microenvironment by modulating osteoclast activity can be a selective approach to regulate cancer metastasis to bone. Along this line, we reported that a neutralizing antibody to PTH-rP, which is a potent local stimulator of osteoclastic bone resorption, also diminished MDA-231 breast cancer metastasis to bone (12). In addition, when TIMP-2 overexpression and ibandronate administration were combined, synergistic suppressive effects on osteolytic bone metastases were accomplished. Thus, our results demonstrate that inhibition of distinct steps in the metastatic process is more effective in suppressing bone metastasis than inhibition of either step alone. In the case of cancer metastasis to soft tissues other than bone, it seems difficult to attempt a combination approach we took here due to the lack of such a unique step as osteoclastic bone resorption. Consequently, the use of anticancer agents which cause a variety of adverse effects is inevitable. On the other hand, in the case of bone metastasis, specific inhibition of osteoclastic bone resorption significantly decreased the development and progression of osteolytic bone lesions without the use of anticancer agents. Furthermore, combination with inhibition of a nonbone related step in metastasis decreased bone metastases. Thus, bone is one of the representative organs in which modulation of the microenvironment makes it possible to impair metastatic cancer cell colonization. Our results support this notion and may provide a unique organ-based therapeutic strategy (4) for breast cancer metastasis in bone.

It has been reported that MMP levels are increased in the plasma of patients with breast cancer (34) and immunohistochemical studies show that breast tumors obtained at biopsy express substantial levels of MMPs (35). Although effects on bone metastasis were not studied, the MMP inhibitor batimastat was found to inhibit breast cancer metastasis to the lung (36). These studies indicate the importance of MMPs in breast cancer phenotype and metastasis. In the present study, we found that MDA-231 cells which form osteolytic bone metastases secreted substantial amounts of MMPs and that overexpression of TIMP-2, a natural inhibitor of MMPs, suppressed MDA-231 cell metastasis to bone. These results indicate that MMPs play a causative role in the development of the osteolytic bone metastases in MDA-231 cells. The mechanism underlying TIMP-2 inhibition of MDA-231 metastasis to bone appears to be due to an inhibition of MDA-231 cell extravasation from the marrow sinus, since MDA-231.TIMP-2/6 showed decreased invasiveness in the transendothelial cancer cell invasion assay using the BMSEC.

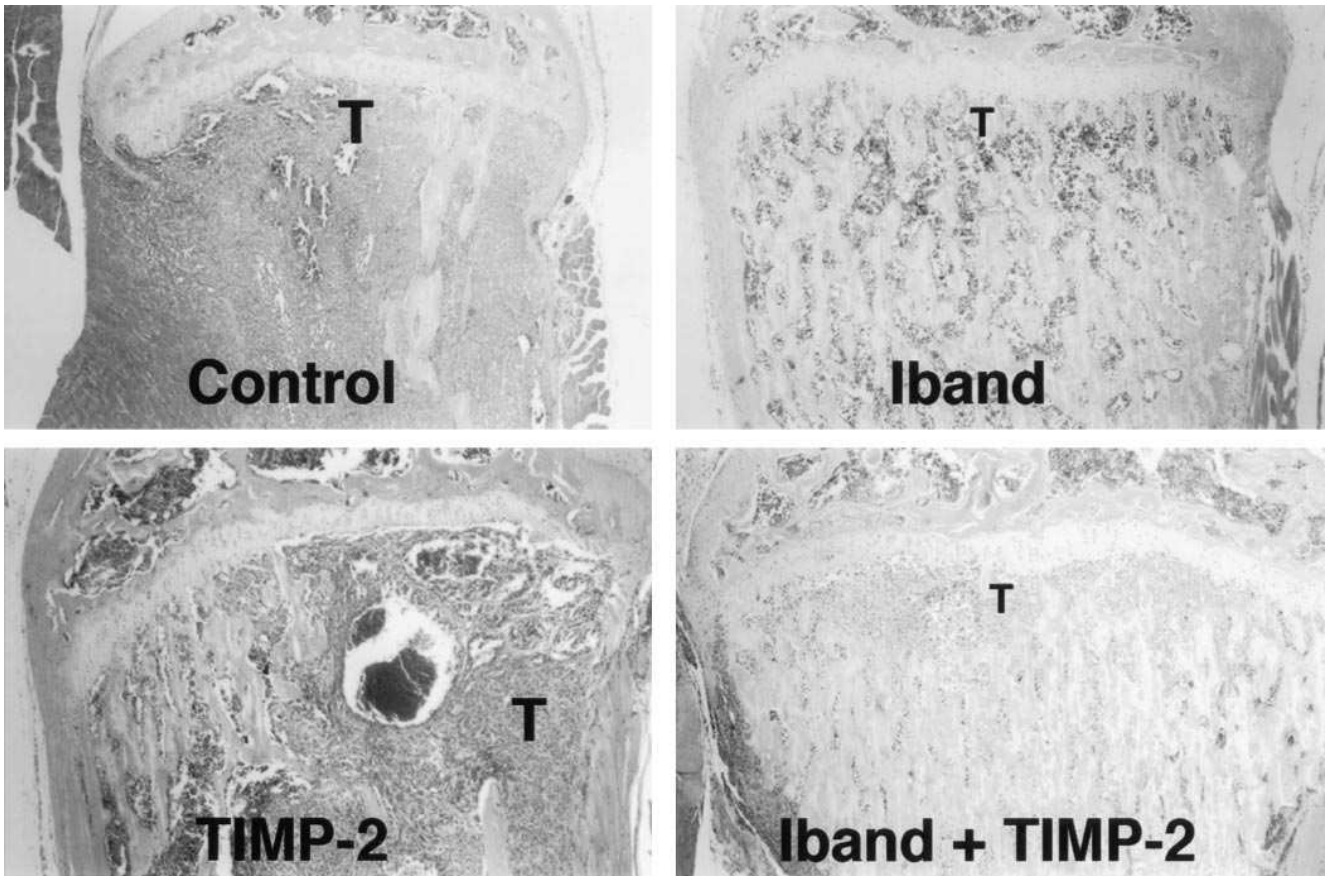

Figure 10. Histology of proximal tibiae. In control mice (*top left*), the bone marrow cavity was occupied by metastatic MDA-231.P cells (*T*) and the marrow trabecular bones were almost completely destroyed. In MDA-231.TIMP-2/6–bearing mice (*bottom left*), colonization of metastatic breast cancer cells in the marrow cavity is localized and necrotic area is observed in the center of the tumor. In ibandronate-treated mice (*top right*), the trabecular bones remained almost intact and metastatic breast cancer burden and osteoclastic bone resorption were markedly decreased. In mice which received both ibandronate administration and MDA-231.TIMP-2/6 cell inoculation (*bottom right*), cancer burden in the marrow cavity was most profoundly reduced. In this experiment, ibandronate was given daily for 3 wk before MDA-231.TIMP-2/6 cell inoculation.

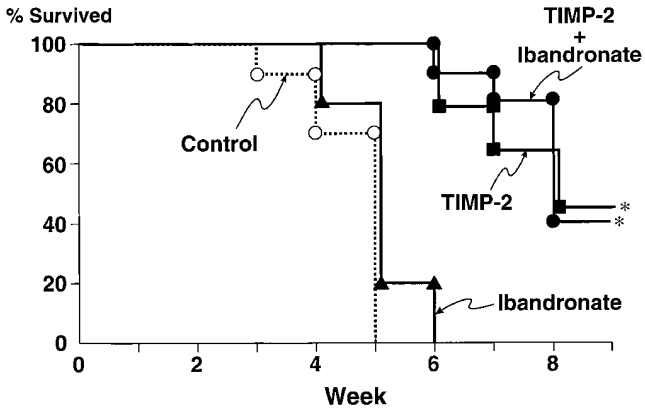

Figure 11. Survival of animals. Experimental protocol was the same as Fig. 9 (see Fig. 6 *C*). Open circle, MDA-231.EV; closed circle, MDA-231.TIMP-2/6; closed triangle, ibandronate; closed square, MDA-231.TIMP-2/6 and ibandronate. Data shown are mean±SEM ($n = 9$ for each treatment group.) *Significantly different from MDA-231.EV or ibandronate alone ($P < 0.01$).

It is notable that MDA-231 cells cultured on bone ECM secreted not only latent forms of MMPs but also activated forms of MMPs. Secretion of an active form of MMP-2 (gelatinase A) by MDA-231 cells cultured on type I collagen, the most abundant ECM in bone, has also been described (37). These results suggest that upon contact with bone, metastatic breast cancer cells may produce large amounts of active MMPs to degrade bone matrices and facilitate their migration, invasion and growth in bone (38). Immunohistochemical study using clinical materials revealed prominent expression of MMP-9 (gelatinase B) in cancer cells housed in bone (39). Furthermore, recent studies showed that MMP-9 (gelatinase B) was involved in the stimulation of bone resorption by parathyroid hormone (40), and that the recombinant TIMP-1 and TIMP-2 inhibited bone resorption (41). Taken together, it is plausible to speculate that MMPs play a role not only in the common steps but also in the specific step of osteoclastic bone destruction in breast cancer metastasis to bone. This may be an additional mechanism of decreased bone metastases in MDA-231.TIMP-2 cells. If this is the case, overexpression of MMP's

natural inhibitor TIMP-2 in breast cancer cells might work as a general and specific inhibitor and be a potentially effective approach for gene therapy of bone metastasis.

In our previous study (13), we noticed that bisphosphonate treatment tended to force metastatic breast cancer cells to escape from bone and cause an increase in tumor volume in surrounding soft tissue in which no osteoclastic bone resorption is required for tumor growth and no accumulation of risedronate occurs. Consequently, it seems likely that colonization of MDA-231 cancer in soft target organs such as lung, liver, and kidney is not interfered by risedronate in tumor-bearing animals. At necropsy, these mice did not show macroscopically evident metastatic foci in these soft organs, but PCR using the primers specific for human house-keeping gene indicates the presence of micrometastases in these soft organs which could cause the death of these animals (data not shown). This may be one reason for the failure of ibandronate to prolong the survival of tumor-bearing animals. Clinically, it has also been demonstrated that bisphosphonate significantly suppresses bone metastases but fails to increase the life span of cancer patients who manifest metastases also in soft organs (42). Thus, it is likely bisphosphonate is unable to suppress cancer colonization in soft tissues. However, in the present study, we found that the tumor burden of MD-231.TIMP-2/6 in the soft tissues surrounding bone was not increased but rather diminished (data not shown). This is presumably due to the suppression of some of the common steps which occur before MDA-231 cell arrest in bone by TIMP-2 overexpression. Thus, use of a combination of inhibitors of both common and specific steps in the bone metastatic process is likely to be more effective than either alone, thereby producing higher chances of abolishing breast cancer metastasis to bone.

In conclusion our results suggest that when therapies are designed based on the unique feature of the bone microenvironment, i.e., osteoclastic bone resorption, in combination with several common steps in the metastatic process, osteolytic bone metastases can be more efficiently and selectively inhibited.

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References

1. Fidler, I.J. 1990. Critical factors in the biology of human cancer metastasis. *Cancer Res.* 50:6130–6138.

2. Ruoslahti, E. 1996. How cancer spreads. *Sci. Am.* 275:72–77.

3. Rusciano, D., and M.M. Burger. 1994. Why do cancer cells metastasize into particular organs. *Bio. Essays.* 14:185–193.

4. Fidler, I.J. 1995. Modulation of the organ microenvironment for treatment of cancer metastasis. *J. Natl. Cancer Inst.* 87:1588–1592.

5. Cifuentes, N., and J.W. Pickren. 1979. Metastases from carcinoma of mammary gland: an autopsy study. *J. Surg. Oncol.* 11:193–205.

6. Coleman, R.E., and R.D. Rubens. 1987. The clinical course of bone metastases from breast cancer. *Br. J. Cancer.* 55:61–66.

7. Yoneda, T., A. Sasaki, and G.R. Mundy. 1994. Osteolytic bone disease in breast cancer. *Breast Cancer Res. Treat.* 32:73–84.

8. Yoneda, T. 1996. Mechanisms of preferential metastasis of breast cancer to bone. *Int. J. Oncol.* 9:103–109.

9. Lewin, D.I. 1996. Evolutions: metastasis. *J. NIH. Res.* 8:85–87.

10. Takeichi, M. 1993. Cadherins in cancer: implications for invasion and metastasis. *Curr. Opin. Cell. Biol.* 5:806–811.

11. Mbalaviele, G., C.R. Dunstan, A. Sasaki, P.J. Williams, G.R. Mundy, and T. Yoneda. 1996. E-cadherin expression in human breast cancer cells suppresses the development of osteolytic bone metastases in an experimental metastasis model. *Cancer Res.* 56:4063–4070.

12. Guise, T.A., J.J. Yin, S.D. Taylor, Y. Kumagai, M. Dallas, B.F. Boyce, T. Yoneda, and G.R. Mundy. 1996. Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J. Clin. Invest.* 98:1544–1549.

13. Sasaki, A., B.F. Boyce, B. Story, K.R. Wright, M. Chapman, R. Boyce, G.R. Mundy, and T. Yoneda. 1995. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. *Cancer Res.* 55:3551–3557.

14. Muhlbauer, R.C., F. Bauss, R. Schenk, J. Janner, E. Bosies, K. Strein, and H. Fleisch. 1991. BM 21.0955, a potent new bisphosphonate to inhibit bone resorption. *J. Bone Min. Res.* 6:1003–1011.

15. Cailleau, R., R. Young, M. Olive, and W.J. Reeves. 1974. Breast tumor cell lines from pleural effusions. *J. Natl. Cancer Inst.* 53:661–674.

16. DeClerck, Y.A., N. Perez, H. Shimada, T.C. Boone, K.E. Langley, and S.M. Taylor. 1992. Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. *Cancer Res.* 52:701–708.

17. Yoneda, T., T.B. Aufdemorte, R. Nishimura, N. Nishikawa, M. Sakuda, M.M. Alsina, J.B. Chavez, and G.R. Mundy. 1991. Occurrence of hypercalcemia and leukocytosis with cachexia in a human squamous cell of the maxilla in athymic nude mice. A novel experimental model of 3 concomitant paraneoplastic syndromes. *J. Clin. Oncol.* 9:468–477.

18. Nakai, M., G.R. Mundy, P.J. Williams, B. Boyce, and T. Yoneda. 1992. A synthetic antagonist to laminin inhibits the formation of osteolytic metastases by human melanoma cells in nude mice. *Cancer Res.* 52:5395–5399.

19. Arteaga, C.L., S.D. Hurd, A.R. Winnier, M.D. Johnson, B.M. Fendly, and J.T. Forbes. 1993. Anti-transforming growth factor (TGF)-β antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-β interactions in human breast cancer progression. *J. Clin. Invest.* 92:2569–2576.

20. Price, J.E., A. Polyzos, R.D. Zhang, and L.M. Daniels. 1990. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res.* 50:717–721.

21. Manicourt, D.H., and V. Lefebvre. 1993. As assay for matrix metalloproteinases and other proteases acting on proteoglycans, casein, or gelatin. *Anal. Biochem.* 215:171–179.

22. Kleiner, D.E., E.J. Unsworth, H.C. Krutzsch, and W.G. Stetler-Stevenson. 1992. Higher-order complex formation between the 72-kilodalton type IV collagenase and tissue inhibitor of metalloproteinases-2. *Biochemistry.* 31:1665–1672.

23. Fridman, R., R.E. Bird, M. Hoyhtya, M. Oelkuct, D. Komarek, C.M. Llang, M.L. Berman, L.A. Liotta, W.G. Stetler-Stevenson, and T.R. Fuerst. 1993. Expression of human recombinant 72 kDa gelatinase and tissue inhibitor of metalloproteinase-2 (TIMP-2): characterization of complex and free enzyme. *Biochem. J.* 289:411–416.

24. Okada T., H. Okuno, and Y. Mitsui. 1994. A novel in vitro assay system for transendothelial tumor cell invasion: significance of E-selectin and α_3 integrin in the transendothelial invasion by HT1080 fibrosarcoma cells. *Clin. & Exp. Metastasis.* 12:305–314.

25. Okada, T., and R.G. Hawley. 1995. Adhesion molecules involved in the binding of murine myeloma cells to bone marrow stromal elements. *Int. J. Cancer.* 63:823–830.

26. Globus, R.K., J. Plouet, and D. Gospodarowicz. 1989. Cultured bovine bone cells synthesize basic fibroblast growth factor and store it in their extracellular matrix. *Endocrinology.* 124:1539–1547.

27. Ray, K.M., and W.G. Stetler-Stevenson. 1995. Gelatinase A activity directly modulates melanoma cell adhesion and spreading. *EMBO J.* 14:908–917.

28. Hayakawa, T., K. Yamashita, E. Ohuchi, and A. Shinagawa. 1994. Cell growth-promoting activity of tissue inhibitor of metalloproteinasaes-2 (TIMP-2). *J. Cell Sci.* 107:2373–2379.

29. Montgomery, A.M.P., B.M. Mueller, R.A. Reisfeld, S.M. Taylor, and Y.A. DeClerck. 1994. Effect of tissue inhibitor of the matrix metalloproteinases-2 expression on the growth and spontaneous metastasis of a human melanoma cell line. *Cancer Res.* 54:5467–5473.

30. Koop, S., R. Khokha, E.E. Schmidt, I.C. MacDonald, V.L. Morris, A.F. Chambers and A.C. Groom. 1994. Overexpression of metalloproteinase inhibitor in B16F10 cells does not affect extravasation but reduces tumor growth. *Cancer Res.* 54:4791–4797.

31. De Bruyn, P.P.H., S. Michelson, and T.B. Thomas. 1971. The migration of blood cells of the bone marrow through the sinusoidal wall. *J. Morph.* 133: 417–438.

32. Wingo, P.A., T. Tong, and S. Bolden. 1995. Cancer statistics, 1995. *CA Cancer J. Clin.* 45:8–30.

33. MacDougall, J.R., and L.M. Matrisian. 1995. Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. *Cancer Metastasis Rev.* 14:351–362.

34. Zucker, S., R.M. Lysik, M.H. Zarrabl, and U. Moll. 1993. M_r 92,000 type IV collagenase is increased in plasma of patients with colon cancer and breast

cancer. *Cancer Res.* 53:140–146.

35. Polette, M., C. Clavel, M. Cockett, S.G. de Bentzmann, G. Murphy, and P. Birembaut. 1993. Detection and localization of mRNAs encoding matrix metalloproteinases and their tissue inhibitor in human breast pathology. *Invasion & Metastasis.* 13:31–37.

36. Sledge, W.G., M. Qulali, R. Goulet, E.A. Bone, and R. Fife. 1995. Effect of matrix metalloproteinase inhibitor batimastat on breast cancer regrowth and metastasis in athymic mice. *J. Natl. Cancer Inst.* 87:1546–1550.

37. Azzam, H.S., G. Arand, M.E. Lippman, and E.W. Thompson. 1993. Association of MMP-2 activation potential with metastatic progression in human breast cancer cell lines independent of MMP-2 production. *J. Natl. Cancer Inst.* 85:1758–1764.

38. Blavier, L., and J.M. Delaisse. 1995. Matrix metalloproteinases are obligatory for the migration of preosteoclasts to the developing marrow cavity of primitive long bones. *J. Cell Sci.* 108:3649–3659.

39. Okada, Y., K. Naka, K. Kawamura, T. Matsumoto, I. Nakanishi, N. Fujimoto, H. Sato, and M. Seiki. 1995. Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/Type IV collagenase=gelatinase B) in osteoclasts: implications for bone resorption. *Lab. Invest.* 72:311–322.

40. Witty, J.P., S.A. Foster, P. Stricklin, L.M. Matrisian, and P.H. Stern. 1996. Parathyroid hormone-induced resorption in fetal rat limb bones is associated with production of the metalloproteinases collagenase and gelatinase B. *J. Bone Min. Res.* 11:72–78.

41. Hill P.A., J.J. Reynolds, and M.C. Meikle. 1993. Inhibition of stimulated bone resorption in vitro by TIMP-1 and TIMP-2. *Biochim. Biophys. Acta.* 1177: 71–74.

42. Hortobagyi, G.N., R.L. Theriault, L. Porter, D. Blayney, A. Lipton, C. Sinoff, H. Wheeler, J.F. Simeone, J. Seaman, R.D. Knight, M. Heffernan, and D.J. Reitsma. 1996. Efficacy of pamidronate in reducing skeletal complications in patients with breast cancer and lytic bone metastases. *N. Engl. J. Med.* 335: 1785–1791.