Matrix metalloproteinase-1 is a crucial bone metastasis factor in a human breast cancer-derived highly invasive cell line

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Received January 19, 2008; Accepted February 25, 2008

DOI: 10.3892/or_00000171

Abstract. Bone metastasis is one of the most severe cancer complications. To analyze the mechanism of bone metastasis, we established highly invasive cell lines from the human breast cancer cell line MDA-MB-231 using an *in vitro* sequential selection system. The cell lines, MDA-231-S10 and MDA-231-S5, were more invasive and more motile than the parental cell line. Moreover, MDA-231-S10 metastasized to bone more often when inoculated into the arterial circulation of nude mice. MDA-231-S10-bearing nude mice had a significantly poorer prognosis, and their bony metastatic tumors grew more rapidly than those of the mice bearing the parental cell line (MDA-231-P). Given that a high expression of matrix metalloproteinase (MMP) is reported to be associated with cancer invasiveness, we examined MMP expression. Our results showed that the expression of MMP-3, -5, -7, -9, -13 and -14 was decreased on Multiplex real-time quantitative RT-PCR analysis in the two new cell lines. The zymographic analysis showed no MMP-2 activity and a decreased MMP-9 activity in MDA-231-S10. However, the expression of MMP-1 in MDA-231-S10 was increased. We therefore concluded that MMP-1 plays a crucial role in breast cancer bone metastasis. Furthermore, our MDA-231-derived cell lines are useful analytical models of MMP-1-associated breast cancer bone metastasis.

Introduction

Bone metastasis frequently occurs in advanced breast cancer patients. Among patients with metastatic breast cancers, 70% have skeletal involvement (1). It has also been observed that patients with predominantly bone metastases have a worse prognosis than those with soft tissue metastases, because the bone metastases are more resistant to endocrine therapy and chemotherapy than metastases to non-bone organs (2,3). Bone metastases cause severe complications, such as intractable bone pain, pathological fractures and hypercalcemia, which can have a largely negative impact on the patient quality of life. Therefore, controlling bone metastases is of major clinical importance.

The mechanisms of breast cancer bone metastasis remain poorly understood. In part, this is due to the lack of a suitable animal bone metastasis experimental model. To be able to analyze the mechanism of bone metastasis, a human cancer cell line that frequently metastasizes to bone is needed. In 1973, MDA-MB-231, a human breast cancer cell line, was established by Cailleau *et al* (4). Since it metastasizes to bone when injected into the arterial circulation of nude mice (5), we postulated that this cell line may contain subpopulations of cells that have differing potentials of metastasizing to bone. Therefore, we attempted to isolate an MDA-MB-231-derived highly invasive breast cancer cell line using a matrigel invasion chamber.

Several *in vivo* and *in vitro* experimental methods have been developed that enable one to isolate subpopulations of cancer cells with a high metastatic potential to a specific organ (6-11). However, these *in vivo* selection systems are time-consuming and expensive. In contrast, our method which uses the matrigel invasion chamber does not require a large investment of time and cost to isolate a cell line that metastasizes to bone. Cancer cells must migrate by crossing multiple extracellular matrix (ECM) barriers, such as the epithelial basement membrane and interstitial stroma, and then enter the blood vessels or lymphatics. Finally, these cells extravasate to form metastatic deposits at a distant site. The cell subpopulation that is isolated using a matrigel invasion chamber is thought to have the specific characteristics that allow the cells to migrate easily by crossing the ECM barrier and then form metastasized foci.

In the present study, we isolated highly invasive cell lines, MDA-231-S5 and MDA-231-S10, from MDA-MB-231 using an *in vitro* selection system and analyzed the new cell lines by comparing them to the parental cell line (MDA-231-P).

Materials and methods

Cell line and culture. MDA-MB-231 was kindly provided by Dr T. Yoneda (Osaka University, Graduate School of Dentistry, Osaka). Cells were cultured to subconfluence in Dulbecco's

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Key words: bone metastasis, breast cancer, invasion, matrix metalloproteinase-1, *in vitro* selection system

modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen Corp., Carlsbad, CA, USA) at 37˚C and 5% CO_2/95% air atmosphere.

Isolation of the highly invasive cell line. To isolate the highly invasive cell line, we used culture insert filters (Becton-Dickinson, Franklin Lakes, NJ, USA) with 8-μm pores, which were precoated with 11 μg of matrigel (Becton-Dickinson) on the upper surface and dried at room temperature overnight. The matrigel was diluted to 0.4 μg/ml with ice-cooled phosphate-buffered saline (PBS) before coating. The upper compartment of the chamber was filled with serum-free medium and the lower one with DMEM containing 10% FBS. The amount of medium was adjusted so that it was the same height in the two chambers. Cells were suspended in serum-free DMEM and seeded on filters of the culture insert at a density of $5x10^5$ cells/chamber. They were incubated at 37˚C for 24 h. Invading cells that appeared on the lower surface of the filters were collected using the EDTA/trypsin technique and cultured in the medium containing 10% FBS. The medium was changed every 2 or 3 days.

Once the collected cells were grown to subconfluence, they were again seeded on filters precoated with matrigel at the same density. This was repeated 10 times. We named cell lines using the prefix S (selection) followed by the number of times the cells had been passed through the matrigel. The cells that invaded the matrigel 5 (MDA-231-S5) and 10 times (MDA-231-S10) were analyzed by comparing them to the parental cell line (MDA-231-P). Two days after seeding on a 60-mm dish, the appearance of each cell line was assessed and photographed using an Olympus IX 70.

Cell growth curve analysis. Once each cell line was grown to subconfluence, the cells were re-seeded (day 0) in a 60-mm dish at a density of $1x10^5$/dish in DMEM supplemented with 10% FBS. The medium was changed every 2 or 3 days. When harvested, the cells were trypsinized and counted 3 times with a hemocytometer (Nitirin, Tokyo) under a phase-contrast light scope. Three dishes for each cell line were counted every other day. The average numbers were plotted and the results were analyzed statistically (post-hoc test).

Assessment of bone metastases. Subconfluent cells were harvested using 0.25% trypsin and 0.05% EDTA after being cultured for 24 h in DMEM supplemented with 10% FBS, and suspended in PBS at a density of $1x10^5$ cells/0.1 ml PBS. The cells were then injected slowly into the left cardiac ventricles of 5-week-old female nude mice (BALB/cA-Jcl-nu, CLEA Japan) under deep anesthesia with pentobarbital (0.05 mg/g). The nude mice were sacrificed 3 weeks after the cardiac injection of the cancer cells and radiographs were taken. The supine mice were laid against the film (Fuji medical X-ray film RX-U, Fuji Film, Japan) and exposed to X-rays at 60 kV for 6 sec using an X-ray exposure system (ED-125L, Shimadzu, Japan). The film was developed using Cepros M2 (Fuji Film, Japan). Osteolytic bone metastases in the upper and lower extremities were assessed based on these radiographs. Using the Lumina Vision image analysis system (Mitani, Japan) the radiographs were analyzed to determine the number and size of the areas of osteolytic bone resorption. All of the procedures were performed according to protocols approved by the committee of animal research of Mie University.

Matrigel invasion assay. Matrigel invasion assay was carried out using filters precoated with matrigel. Cells were suspended in serum-free DMEM and seeded on filters precoated with matrigel (11 μg/filter) at a density of $5x10^5$/chamber. The lower compartment of the chamber was filled with DMEM containing 10% FBS. The amount of medium was adjusted to the same height in the two chambers. The cells were allowed to migrate for 24 h at 37˚C in a humidified atmosphere containing 5% CO_2. The cells on the upper side of the filters were removed mechanically. The filters were fixed in 70% ethanol. The cells that had migrated to the lower surface of the filters were stained with 0.4% trypan blue and counted using a phase-contrast light microscope. The results were statistically analyzed. Four chambers were used for this assay, and 5 areas in each chamber were investigated.

Phagokinetic track assay. A phagokinetic track assay was performed to quantitatively analyze cell motility. Matrigel was diluted with ice-cooled PBS to 10 μg/ml for the purpose of coating. Coverslips were immersed in the diluted matrigel and incubated at 37˚C for 1 h. Colloidal gold suspension was prepared by adding 33 ml distilled water and 18 ml Na_2CO_3 (36.5 mM) to 5.4 ml $AuHCl_4$ (14.5 mM). The mixture was heated to 100˚C and 5.4 ml of 0.1% formaldehyde solution was added. The coverslips were placed in dishes and the hot gold particle suspension was layered on top. The following day, the gold particle-coated coverslips were sterilized by exposure to UV light for 10 min. The coverslips were washed twice with PBS to remove any unattached gold particles and placed in 35-mm dishes. Two thousand cells, suspended in DMEM supplemented with 10% FBS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin, were plated in the dishes and incubated for 12 h. The phagokinetic tracks were observed on light microscopy. The images were imported into the computer, and the area of the phagokinetic tracks was analyzed using the Lumina Vision image analysis system. The result was statistically analyzed. Three dishes were used for this assay, and 5 tracks were observed in each dish.

Zymographic analysis. The gelatinase activity of the conditioned medium was tested by zymography using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 mg/ml gelatin.

A total of $1x10^6$ cells suspended in DMEM supplemented with 10% FBS was seeded on 60-mm plastic dishes at 37˚C. The following day, the media were changed to 2 ml of serum-free medium and incubated at 37˚C for 24 h. The supernatant of the conditioned medium was collected and centrifuged to remove cell debris before further use. The conditioned medium from the MDA-231-derived cell lines was mixed with a non-reducing sample buffer (0.3 M Tris-HCl, pH 6.8, 12% SDS and 0.00012% bromophenol blue) at a ratio of 1:5 and incubated at room temperature for 30 min. Then the samples were electrophoresed on 10% polyacrylamide gel containing gelatin (Sigma-Aldrich Japan, Tokyo) (1 mg/ml) in the running gel at a constant 50 mA. The sample volumes were strictly

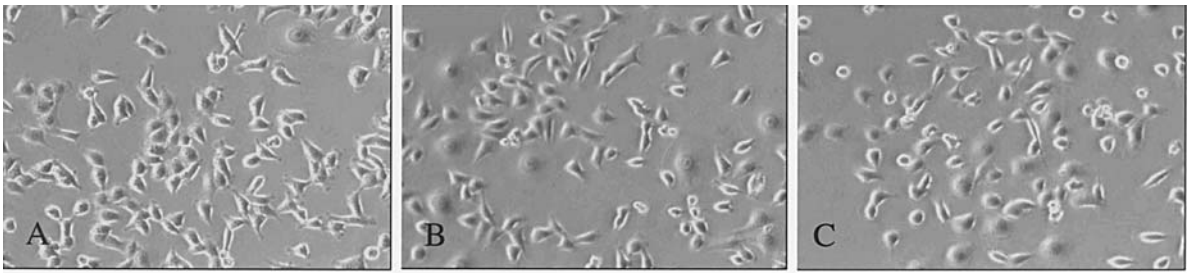

Figure 1. Morphological appearance of parental and newly established cell lines. No morphological differences were noted in the appearance of MDA-231-P (A), MDA-231-S5 (B) and MDA-231-S10 (C) when cultured on the plastic dishes.

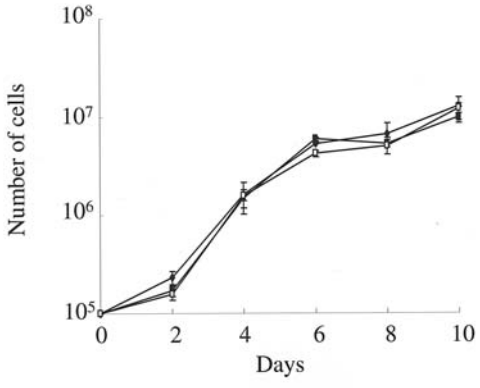

Figure 2. Growth rates of parental and newly established cell lines. Cells were seeded on day 0 and counted every 2 days. No difference in the cell growth between the 3 cell lines of MDA-231-P (♦), MDA-231-S5 (■) and MDA-231-S10 (□) existed. Values represent means ± SD (n=3 per group).

controlled to be the same (50 μl). After electrophoresis, the gel was rinsed three times with washing buffer (10 mM Tris-HCl, pH 7.5 and 2.5% Triton X-100) at room temperature for 30 min to remove the SDS. After being washed twice with the metalloproteinase buffer for 30 min, the gel was incubated with this buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ and 0.05% NaN_3) at 37˚C for 24 h. It was then stained with 0.1% Coomassie brilliant blue staining solution [0.1% Coomassie brilliant blue 250-R (Sigma-Aldrich Japan), 10% acetic acid and 10% isopropanol] for 30 min and destained in 10% acetic acid and 30% methanol. This assay was carried out three times.

Analysis of PTH-rP production. The parathyroid-related protein (PTH-rP) concentrations in the cell culture supernatant of each MDA-231-derived cell line were analyzed using a competitive enzyme immunoassay kit (Peninsula Laboratories, San Carlos, CA, USA). The assay was performed according to the manufacturer's protocol. The supernatant in 4 dishes for each cell line was observed. The result was statistically analyzed using the Kruskal-Wallis test. This assay was carried out twice.

RNA extraction and cDNA synthesis. The total RNA from the subconfluent cells grown in plastic dishes was extracted using an mRNA purification kit (Quick Prep Micro, Amersham Pharmacia Biotech), according to the manufacturer's protocol. The RNA was reverse transcribed using an oligo (dT) primer according to the manufacturer's protocol (SuperScript first-strand synthesis system for RT-PCR, Invitrogen). The cDNA was diluted to twice the original volume and then stored at -80˚C.

Multiplex real-time quantitative RT-PCR analysis. The matrix metalloproteinase (MMPs) expression was measured by real-time quantitative RT-PCR, based on the Taq man methodology, using the ABI PRISM 7000 sequence detection system (Applied Biosystems Japan, Tokyo). MMP-1 (collagenase-1), MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-9 (gelatinase B), MMP-13 (collagenase-3) and MMP-14 (MT1-MMP) were investigated. All of the reagents were obtained from Applied Biosystems. The PCR mixture consisted of 25 μl of Taq man universal master mix, 2.5 μl of assay-on-demand gene expression assay mix and 22.5 μl of cDNA diluted in distilled water. The PCR reactions were carried out in a 96-well microtitre plate, using the procedure: 1 cycle at 50˚C for 2 min, 1 cycle at 95˚C for 10 min and 50 cycles at 95˚C for 15 sec and 60˚C for 1 min. The relative gene expression levels were calculated using standard curves generated by serial dilutions of cDNA obtained from MDA-231-P. Using SDS software (Version 1.0), the relative expression amounts of the samples were calculated compared to the expression levels determined by the standard curve. The expression levels of the housekeeping gene, ß-actin, were quantitated in the same way. The expression levels of each gene and sample were divided by the ß-actin expression level. All mRNA levels were determined in relation to MDA-231-P. All of the samples were analyzed in triplicate.

Results

Morphology and cell proliferation. After the establishment of the two MDA-231-derived cell lines, we investigated whether any morphological differences between the cell lines existed. No morphological differences were noted among MDA-231-P, MDA-231-S5 and MDA-231-S10 when they were cultured on the plastic dishes (Fig. 1). The growth rates were not statistically different between the cell lines (Fig. 2).

Survival of MDA-231-derived cell line bearing nude mice. To evaluate the biological aggressiveness of our established cell lines, we used the heart injection method and observed the survival rates of nude mice bearing our established MDA-231-derived cell line, MDA-231-S10, and those bearing the MDA-231-P cell line.

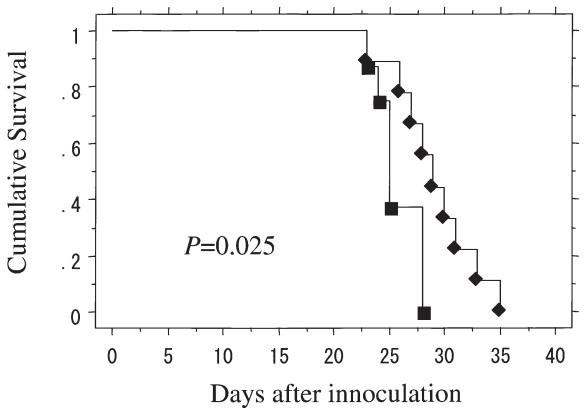

Figure 3. Kaplan-Meier survival analysis of MDA-231-derived cell line bearing nude mice. Cells ($1x10^5$) suspended in 0.1 ml PBS were injected into the left cardiac ventricles of anesthetized 5-week-old female nude mice. The average survival of MDA-231-P-bearing nude mice was 29 days, while that of MDA-231-S10-bearing nude mice was 26 days. The Kaplan-Meier survival analysis showed a lower survival in MDA-231-S10-bearing nude mice than in the MDA-231-P-bearing nude mice (P=0.025). [MDA-231-P (♦), n=9 and MDA-231-S10 (■), n=8].

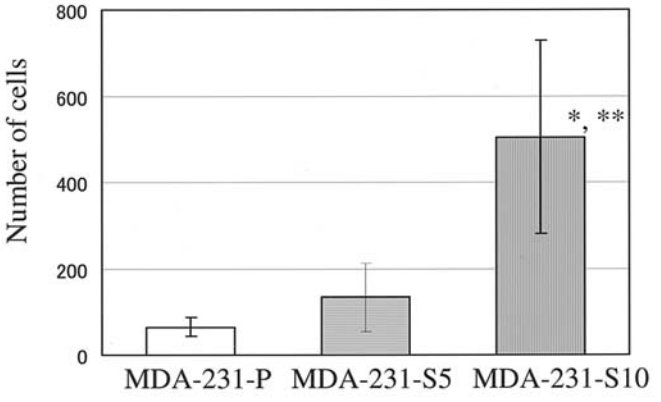

Figure 5. Matrigel invasion assay of parental and newly established cell lines. The matrigel invasion assay was carried out using filters of the culture insert precoated with matrigel. Cells were suspended in serum-free DMEM and seeded on filters precoated with matrigel at a density of $5x10^5$/chamber. The lower compartment of the chamber was filled with DMEM containing 10% FBS. The cells were incubated for 24 h. Invading cells were stained and counted. MDA-231-S10 was significantly more invasive than MDA-231-S5 and MDA-231-P. Values represent means ± SD (n=20 per group). $^{*,\ **}$P<0.05 compared with MDA-231-P and MDA-231-S5, respectively.

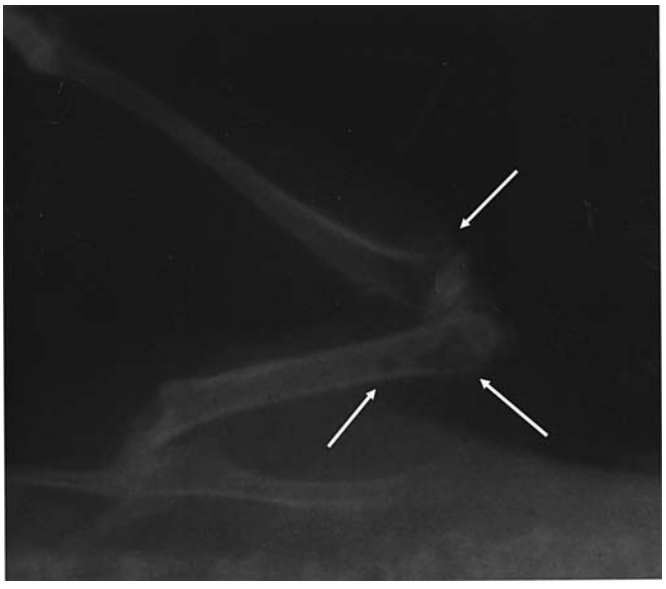

Figure 4. Bone metastases of an MDA-231-S10-bearing nude mouse. The radiograph shows the left hind limb of an MDA-231-S10-bearing nude mouse. The arrows indicate osteolytic bone metastases. Three osteolytic bone metastases are clearly shown.

The mice died 23-35 days after inoculation. The average survival of the MDA-231-P-bearing nude mice was ~29 days, and the average survival of the MDA-231-S10-bearing nude mice was ~26 days. Survival rate analysis based on the Kaplan-Meier method, showed that the survival of MDA-231-S10-bearing nude mice was significantly shorter than that of the MDA-231-P bearing nude mice (P=0.025) (Fig. 3).

Bony metastatic potential of our established MDA-231-derived cell line. Since we had postulated that our established cell lines would have a high metastatic potential, we compared their bony metastatic potential by using the intracardiac inoculation method. Nude mice were sacrificed three weeks after cardiac injections of tumor cells, and radiographs were taken to evaluate bone metastases. Using the Lumina Vision image analysis system the film was analyzed to determine the number and size of the areas with osteolytic bony resorption. The most frequent bone metastasis sites were the distal femur and proximal tibia (Fig. 4). Severe bone metastases causing pathological fractures were observed at these sites. We observed no metastases in the skull, ribs, spine and pelvis. In the upper and lower extremities, MDA-231-P-bearing nude mice had 1.8±1.8 osteolytic metastases, and MDA-231-S10-bearing nude mice had 3.6±2.4 osteolytic metastases (P=NS for the comparison). The total area of the bone metastases in MDA-231-P-bearing nude mice was 2.29±2.36 mm^2, while in MDA-231-S10-bearing nude mice it was 8.19±5.34 mm^2. The mean area of each metastasis was 0.84±0.77 mm^2 in MDA-231-P-bearing nude mice and 2.28±0.71 mm^2 in MDA-231-S10-bearing nude mice. MDA-231-S10-bearing nude mice had statistically larger bone metastases (Table I).

Matrigel invasion assay. To clarify the reason why there was a difference in the bony metastatic capacity between MDA-231-P and MDA-231-S10, we initially examined the matrigel invasion capacity of each cell line. The matrigel invasion assay showed that MDA-231-S10 was significantly more invasive than MDA-231-S5 and MDA-231-P. MDA-231-S5 tended to be more invasive than MDA-231-P, but there was no statistically significant difference (Fig. 5).

Phagokinetic track assay. To evaluate the motility of each cell line, we used a phagokinetic track assay (12) (Fig. 6a). MDA-231-S10 was significantly more motile than MDA-231-P (P<0.05, n=15), and MDA-231-S5 was significantly more motile than MDA-231-P (P<0.05, n=15). Though MDA-231-S10 tended to be more motile than MDA-231-S5, the difference was not statistically significant (Fig. 6b).

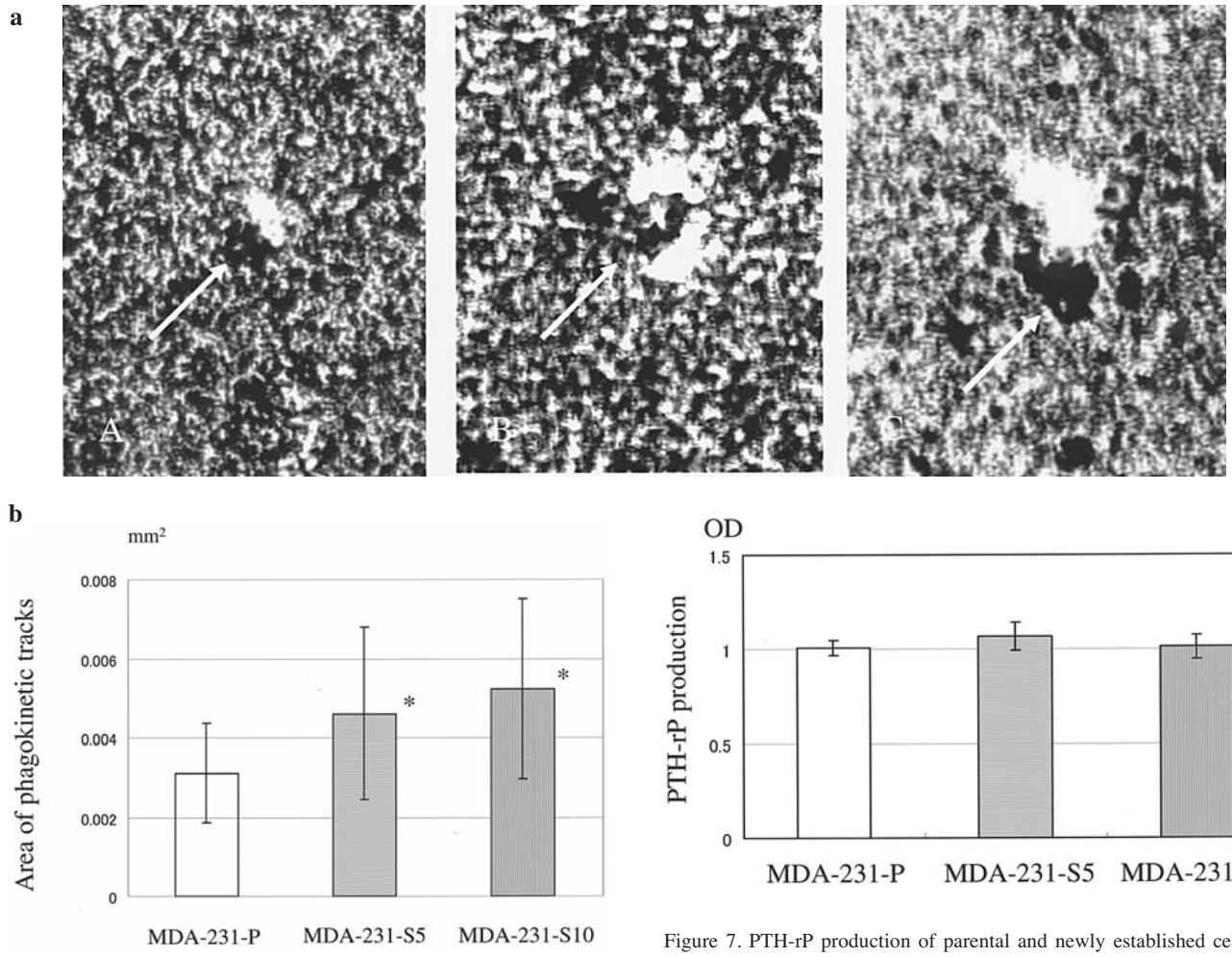

Figure 6. (a) Microscopic findings of phagokinetic tracks of parental and newly established cell lines. A phagokinetic track assay was performed to quantitatively analyze cell motility. A total of 2000 cells suspended in DMEM supplemented with 10% FBS were plated on the coverslips precoated with hot gold particle suspension and incubated for 12 h. The areas of phagokinetic tracks were investigated with light microscopy. The black spots and arrows indicate cells while the white particle-free area around the cell is the phagokinetic area. (A) MDA-231-P, (B) MDA-231-S5 and (C) MDA-231-S10. (b) The phagokinetic track assay of parental and newly established cell lines. The areas of phagokinetic tracks were measured using the Lumina Vision image analysis system. MDA-231-S10 and MDA-231-S5 cells were significantly more motile than MDA-231-P cells. The MDA-231 cells became more motile through our selection process. Values represent means ± SD (n=15 per group). *P<0.05, compared with MDA-231-P.

Figure 7. PTH-rP production of parental and newly established cell lines. PTH-rP production in MDA-231-P, MDA-231-S5 and MDA-231-S10 was investigated using a competitive enzyme immunoassay. No significant differences among the 3 cell lines existed. Values represent means ± SD (n=4 per group).

PTH-rP production. To investigate whether the production of PTH-rP was associated with metastatic properties in our established cell lines, we examined the expression of PTH-rP in each cell line. PTH-rP production was not significantly different among MDA-231-P, MDA-231-S5 and MDA-231-S10 (Fig. 7).

Zymographic analysis. Since MMP activities are required when cells migrate through the matrigel barrier, we investigated MMP activities using gelatin zymography. The zymographic analysis showed 90 kDa bands, which represented the activity of MMP-9. Bands of 72 kDa which represented the activity of MMP-2 were not detected. Contrary to our expectations,

Table I. *In vivo* bony metastatic analysis.

	Cell lines		
	MDA-231-P	MDA-231-S10	P-value
Number of bone metastases	1.80±1.80	3.60±2.40	NS
Total area of bone metastases[a]	2.29±2.36	8.19±5.34	0.0142
Mean area of each bone metastasis[b]	0.84±0.77	2.28±0.71	0.0067

In vivo bony metastatic analysis of parental and newly established cell lines. Three weeks after cardiac injection, radiographs were taken. Using the Lumina Vision image analysis system, the film was analyzed to quantify the number and size of the osteolytic bone resorption areas. MDA-231-derived highly invasive cell line bearing nude mice had a significantly larger area of metastasis. Values represent means ± SD (MDA-231-P, n=9 and MDA-231-S10, n=8). [a]P<0.05, compared with MDA-231-P and [b]P<0.01, compared with MDA-231-P. NS, not significant.

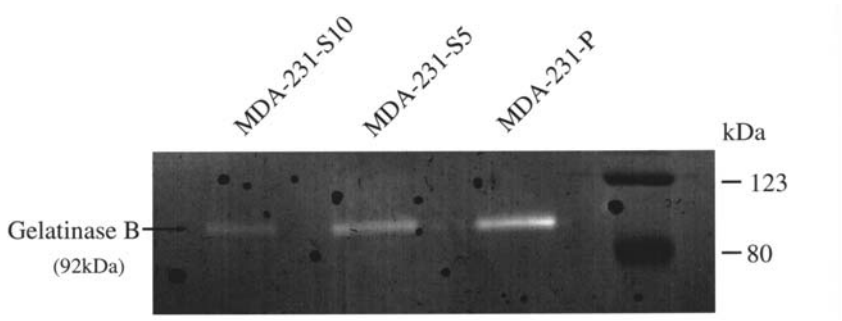

Figure 8. Gelatin zymography of parental and newly established cell lines. Gelatinase activities of a conditioned medium were tested by gelatin zymography using SDS-PAGE containing 1 mg gelatin/ml. The zymographic analysis showed 90 kDa bands which represented the activities of MMP-9. Bands of 72 kDa which represented the activities of MMP-2 were not detected. MDA-231-S10 had the lowest gelatinase activity, while MDA-231-P had the highest.

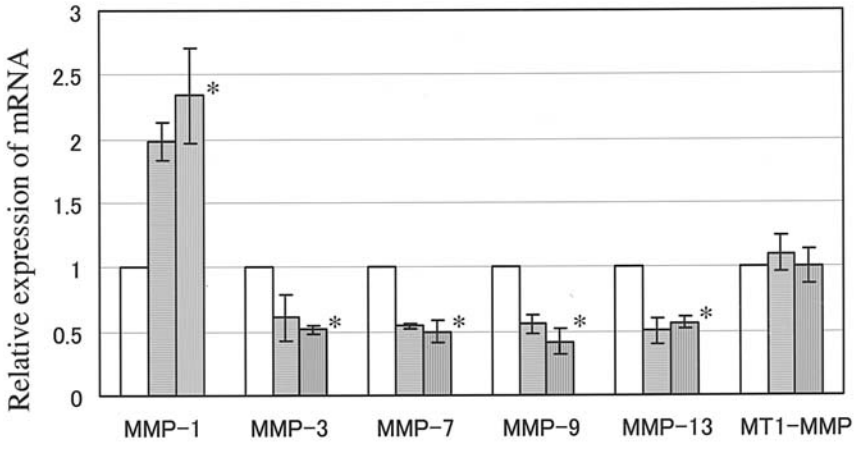

Figure 9. Multiplex real-time quantitative RT-PCR analysis of the MMP expression in parental and newly established cell lines. Multiplex real-time quantitative RT-PCR analysis of mRNA from cells grown in plastic plates was performed to quantitatively analyze MMP expression. MDA-231-S5 (laterally striped bar) and MDA-231-S10 (vertically striped bar) were normalized to MDA-231-P (white bar), which is given a value of 1. MMP-1 expression was increased in the process of selecting subpopulations of cells with a high potential for bony metastases. MDA-231-S10 had an ~2.3 fold higher MMP-1 expression than MDA-231-P. Contrary to our expectations, MMP-3, -7, -9 and -13 expression was significantly decreased. MT1-MMP expression remained unchanged. Values represent means ± SD (n=3 per group). $^{*}P<0.05$, compared with MDA-231-P.

MDA-231-P had the highest gelatinase activity, and MDA-231-S10 the lowest (Fig. 8).

Matrilysin, MMP-7, activity was also examined using casein zymography with concentrated supernatants. However, caseinolytic activity was not detected (data not shown).

Multiplex real-time quantitative RT-PCR analysis. To clarify MMP participation with respect to the different bony metastatic properties of the three cell lines, we examined the expression of various types of MMP transcripts (Fig. 9). Real-time quantitative RT-PCR analysis showed that MDA-231-S10 and MDA-231-S5 had a significantly higher MMP-1 expression than did MDA-231-P. MDA-231-S10 had an ~2.3-fold higher MMP-1 expression than did MDA-231-P. Contrary to our expectations, this analysis also showed that the expression of MMP-3, -7, -9 and -13 transcripts was decreased by the process of selecting subpopulations with a high bony metastatic potential. MT1-MMP expression remained unchanged. MMP-2 expression was not investigated because it was not detected on gelatin zymography. These results suggest that MMP-1 may play an important role in maintaining the high bony metastatic property of MDA-231-S10.

Discussion

Advanced multidisciplinary treatment, which includes surgery, chemotherapy and radiotherapy prolong the survival of cancer patients. Nevertheless, cancer metastasis control remains a challenge because the mechanism of cancer metastasis is complicated and poorly understood. Thus, cancer treatment has to deal with the control of primary cancers and management of metastatic lesions.

In particular, bone metastases can cause severe complications, including intractable bone pain, pathological fractures and hypercalcemia, all of which greatly impair the patient quality of life. Animal model research into bone metastasis is starting to play an important role in dealing with this challenge. Several *in vivo* and *in vitro* experimental methods have been developed that allow for the isolation of subpopulations of cancer cells that have a high metastatic potential to specific organs (6-11). However, *in vivo* selection systems are time-consuming and expensive. In contrast, our method which uses a matrigel invasion chamber to isolate a cell line that metastasizes to bone does not require a large investment of time and money.

Our established MDA-231-derived cell lines showed no differences in morphology and growth rates *in vitro*. However, when they were inoculated into the left cardiac ventricles of nude mice, MDA-231-S10 grew more rapidly than MDA-231-P at the sites of bony metastases, although the number of bone metastases did not increase. These results indicate that there was a difference in the bony metastastic potential between the two cell lines, which was likely the result of different growth activity of the tumor cells within the intramedullary bony microenvironment. Studies have shown that a variety of growth factors produced by osteoblasts, including insulin-like growth factors (IGFs), transforming growth factors (TGF) α and ß, fibroblast growth factors and bone morphogenetic proteins are subsequently stored in the bone matrix (13,14). These growth factors are released in their active form into the bone marrow when bone is degraded during osteoclastic bone resorption (15). Therefore, it is possible that the difference in growth seen with the MDA-231-P and MDA-231-S10 cell lines may be explained by the two cell lines having different sensitivities to the growth factors released at the metastatic site due to osteoclast-mediated bone resorption.

Furthermore, our established cell lines were more motile and more invasive than the parental cell line. Rho protein and Rho-regulatory proteins are key components that mediate breast cancer motility (16). While we have yet to examine Rho GTPase signaling in our cell lines, such factors may contribute to the difference in motility between MDA-231-P and MDA-231-S10 at bony metastatic sites.

Reports have suggested that the MMPs are involved in the initial step of ECM degradation during the metastatic process (17-23). MMPs have a more complex role than was initially appreciated, in that the MMP-mediated degradation of ECM components may generate inhibitors of angiogenesis, as well as products that stimulate cell migration and/or invasion and release activated cytokines stored in ECM (24,25). To clarify the role of MMPs in our established cell lines with their different bony metastatic potentials, we examined MMP-2, -7 and -9 activities using gelatin and casein zymography, and studied the expression of transcripts of MMP-1, -3, -7, -9, -13 and -14. MDA-231-S10 and MDA-231-S5 had a significantly higher MMP-1 expression than MDA-231-P. Kang *et al* used microarray analysis to show that overexpression of MMP-1 enhances MDA-231 bone metastases (26). In human breast carcinoma, the expression of MMP-1 has been investigated at the primary (27-30) and bony metastatic sites (31). In bone matrix, type I collagen is the major protein, comprising of ~90% of the organic matrix (32). During cancer dissemination, the bone matrix must be broken down mainly by the interstitial collagenase, MMP-1. Our results suggest that MMP-1 may play an important role in maintaining the high bony metastatic potential in our established cell line, MDA-231-S10. It appears that our *in vitro* selection system allows for the selection of a cell line with high MMP-1 activity, and is a suitable model for elucidating the MMP-1-related mechanism of bone metastasis.

Contrary to our expectations, our analysis also showed that the expression or activity of transcripts of MMP-3, -7, -9 and -13 decreased in the process of selecting cell subpopulations with a high potential for bony metastases. However, MMP-14 (MT1-MMP) expression remained unchanged. The major components of reconstituted matrigel include: laminin (60%), type IV collagen (30%), heparin sulfate proteoglycan (3%), entactin (1%) and nidogen (5%) (33). The type IV collagen component of the basement membrane is thought to be degraded mostly by MMP-2 and -9. Surprisingly, our results showed that a higher invasive capacity was associated with a lower expression of MMP-9. The reason for this negative correlation is uncertain, though degradation of the matrigel may be performed by proteinases other than MMP-9 by the cells chosen in our selection system. Kawamata *et al* also reported a decreased expression of MMP-9 in a highly invasive esophageal squamous cell carcinoma line that was isolated by an *in vitro* selection system similar to ours (34). Their findings are consistent with ours. Furthermore, Farina *et al* reported that MMP-3 inhibited MDA-231 cell invasion *in vitro* by a mechanism involving plasminogen degradation to fragments that limit the degradation of laminin, the major structural component of reconstituted matrigel (35). Thus, this report supports our finding that a higher invasive potential was associated with a lower expression of MMP-3.

The survival rate of MDA-231-S10-bearing nude mice was significantly lower than that of MDA-231-P-bearing nude mice. Moreover, MDA-231-S10-bearing nude mice had a markedly cachexic appearance (data not shown). Although at first we suspected the existence of hypercalcemia in MDA-231-S10 mice, there was no evidence of hypercalcemia. Unknown factors may have enhanced the progression of cachexia in MDA-231-S10-bearing nude mice, since the reasons for the short survival of MDA-231-S10-bearing nude mice remain uncertain.

Cancer invasiveness and metastatic potential are also determined by the expression levels of certain cytokines. Many researchers have reported that osteoclasts stimulated by cancer cells either directly or indirectly play an important role in increasing bone resorption (36-39). In breast cancer, PTH-rP secreted by cancer cells has been shown to stimulate the formation and resorptive activity of osteoclasts (17,40,41). However, our results showed that PTH-rP production by the MDA-231-S10 was not up-regulated despite an increased bone resorption capacity. These data indicate that the production of PTH-rP is not correlated with the osteolytic bone metastasis in our *in vivo* animal model. Sanchez-Sweatman *et al* reported the possibility that metastatic cancer cells degrade bone matrix directly and that this is partially mediated by MMPs (42), which is consistent with our results.

In conclusion, we isolated highly invasive and metastatic cell lines *in vitro* with a sequential passage method using filters of the culture insert precoated with matrigel. The two cell lines had the same appearance and growth rates, in the plastic dishes, as the parent cell line. When inoculated into the left ventricles of nude mice, the two cell lines metastasized to bone to a greater extent than the parent cell line. The two cell lines had a higher MMP-1 expression than the parent cell line. The results of the present study suggest that MMP-1 may play a crucial role in the formation of bone metastases caused by MDA-231-S10 cells injected into our animal model. Our MDA-231-derived cell lines will become powerful tools in the study of breast cancer bony metastases.

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