Cadherin-11-mediated interactions with bone marrow stromal/osteoblastic cells support selective colonization of breast cancer cells in bone

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Abstract. Cell adhesion molecules have been implicated in the selective colonization of cancer in distant organs. Breast cancer has a strong predilection for spreading to bone. Cadherin-11, which is one of the classical type-2 cadherin family members and mediates homophilic cell-cell adhesion, is constitutively expressed in stromal and osteoblastic cells in bone marrow. Elevated cadherin-11 expression is also found in aggressive human breast cancers. Here, we investigated the role of the interactions between breast cancer cells and bone marrow stromal/osteoblastic cells via cadherin-11 in the selective spread to bone. The bone-seeking clone of the MDA-MB-231 human breast cancer cells showed greater cadherin-11 expression than the parental and the brain-seeking clone. Cadherin-11 overexpression in MDA-MB-231 cells increased bone metastases with promoted bone resorption, while the natural variant form of cadherin-11 that is unable to establish cell-cell adhesion did not. Of note, introduction of cadherin-11 showed no effects on lung metastases. Fluorescence-activated cell sorter analysis using the fluorescent dye-labeled cancer cells showed that early colonization in bone marrow was increased by cadherin-11. Co-cultures with the MC3T3-E1 osteoblastic cells that constitutively expressed cadherin-11 caused an up-regulation of parathyroid hormone-related protein (PTH-rP) production in MDA-MB-231 cells overexpressing cadherin-11. The conditioned medium of the co-cultures increased osteoclastogenesis, which was blocked by a neutralizing antibody to PTH-rP. In conclusion, our results suggest that cadherin-11 promotes homing and migration to bone and osteoclastogenesis through mediating the homophilic interactions of breast cancer cells with marrow stromal/osteoblastic cells, thereby enhancing bone metastases.

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Introduction

Bone is one of the most preferential sites of cancer metastases (1-3). Cancers including breast, prostate and lung cancers have a strong predilection for spreading to bone. Although the precise molecular mechanism underlying the preferential metastasis of these cancers to bone is yet to be elucidated, cell adhesion molecules (CAMs) present in bone have been proposed to play a supportive role in bone-selective metastasis of cancers (4-6).

Cadherins are transmembrane Ca^{2+}-dependent CAMs that mediate the homophilic cell-cell adhesion (7,8) and have been implicated in cancer invasion and metastasis (9,10). Cadherin-11 is one of the classical type 2 cadherin family members originally isolated from the mouse osteoblastic cell line MC3T3-E1. Cadherin-11 was specifically and inherently expressed in bone marrow stromal cells and osteoblasts (11). Of note, cadherin-11 expression was also detected in human breast cancers (12) and expression levels of cadherin-11 were correlated with the aggressiveness of breast cancers (12). It is widely recognized that breast cancer preferentially disseminates to bone (1-3). These earlier observations led us to hypothesize that cadherin-11 expressed in bone marrow stromal cells/osteoblasts supports the selective colonization of breast cancer cells in bone through interacting with cadherin-11 expressed in breast cancer cells in a homophilic manner.

To approach this, we established the MDA-MB-231 human breast cancer cells stably transfected with an intact and a naturally-occurring splice variant form of cadherin-11 cDNA (MDA/Cad11 and MDA/Var, respectively). The variant form of cadherin-11 is unable to establish cell-cell interactions due to a lack of one third of the transmembrane and the entire cytoplasmic domain (13,14). We then examined the capacity of these transfectants to metastasize to bone compared with the MDA-MB-231 parental cells (MDA/Pa) in a well-characterized animal model of bone metastasis (15,16). We found that bone metastases were significantly increased in MDA/Cad11 compared with MDA/Pa, while MDA/Var

showed less bone metastases than MDA/Pa. Of note, however, MDA/Cad11 showed no changes in lung metastases. Our results suggest that cadherin-11 plays a critical role in the preferential colonization of breast cancer cells in bone.

Materials and methods

Reagents. Mouse monoclonal antibody to cadherin-11, which recognizes the intact form of cadherin-11, and goat polyclonal antibody to OB-cadherin-2, which recognizes the variant form of cadherin-11, were purchased from Zymed Laboratories Inc. (South San Francisco, CA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Neutralizing antibody to human parathyroid hormone-related protein (PTH-rP) was kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals used in this study were purchased from Sigma-Aldrich (St Louis, MO) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise described.

Cells. The human breast cancer cell line MDA-MB-231 (American Type Culture Collection, Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplement with 10% fetal bovine serum (FBS; Asahi Glass Techno Corp., Tokyo, Japan) and kanamycin sulfate (250 μg/ml, Meiji Seika Kaisha, Ltd., Tokyo, Japan). The bone- and brain-seeking clones of MDA-MB-231 cells (MDA-231BO and MDA-231BR, respectively) were established as described previously (15). The mouse fibroblastic cell line NIH3T3-3 (Riken BioResource Center, Ibaragi, Japan) and the mouse osteoblastic cell line MC3T3-E1 (Riken BioResource Center) were cultured in α-minimum essential medium (αMEM; Sigma-Aldrich) plus 10% FBS and kanamycin sulfate. All cells were maintained in a humidified atmosphere of 5% CO_2 in air.

Transfection. Both intact and variant forms of cadherin-11 cDNA in pCXN2 vector (kindly provided by Dr Akira Kudo, Tokyo Institute of Technology, Kanagawa, Japan) (10,13), were transfected into the parental MDA-MB-231 cells (MDA/Pa) using FuGENE 6 Transfection Reagent (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer's protocol. Colonies resistant to 1 mg/ml G418 (Sigma-Aldrich) were isolated and cloned. The transfectants were designated MDA/Cad11 and MDA/Var, respectively.

Immunoprecipitation and immunoblotting. Cells were lysed in the lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, and protein phosphatase inhibitors including 1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, and 100 μM sodium orthovanadate). Cell lysates were incubated with a primary antibody, followed by immunoprecipitation with protein G PLUS-agarose (Santa Cruz Biotechnology). Immunoprecipitates were washed four times with lysis buffer, boiled in SDS sample buffer and centrifuged. The supernatants or whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with corresponding antibodies, and visualized with horseradish peroxidase coupled to protein A (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) or horseradish peroxidase coupled with anti-mouse IgG antibody (Cappel Biochemical Division, ICN Pharmaceuticals Inc., Aurora, OH) with enhancement by chemiluminescence using Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products Inc., Boston, MA). We confirmed that equal amounts of proteins were loaded by staining the transferred membranes with Ponceau S.

Cell adhesion assay. NIH3T3-3 or MC3T3-E1 cells were plated in 12-well plates and fixed with 0.2% formaldehyde in PBS at confluency. The MDA-MB-231 clones ($5x10^4$ cells) labeled for 12 h with Vybrant CFDA SE Cell Tracer Kit (V-12883; Molecular Probes, Inc., Eugene, OR) were plated, incubated on the fixed cell layer for 1 h, and washed three times with PBS to remove unattached cells. Tumor cell adhesiveness was defined as the mean cell number of fluorescently labeled cancer cells in 5 microscopic fields at magnification x400.

Animal experiments

Mice. Four-week-old female athymic nude mice (Japan SLC, Inc., Shizuoka, Japan) were used. The number of mice used in each experiment is described in the figures. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry before experiments were started.

Bone metastasis. MDA-MB-231 cells ($1x10^5$ cells) suspended in 0.1 ml of PBS were injected with a 27-gauge needle into the left cardiac ventricle of nude mice under anesthesia with pentobarbital (0.05 mg/g of body weight, Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) as described previously (16).

Lung metastasis. MDA-MB-231 cells ($1x10^6$ cells/mouse/0.1 ml PBS) were inoculated through a lateral tail vein of nude mice under anesthesia with pentobarbital (0.05 mg/g of body weight).

Radiographical examination. The number and area of osteolytic lesions were determined on radiographs 28 days after the cancer cell inoculation as described previously (16). Radiographs were carefully evaluated using image analysis software (Image Pro Plus; Media Cybernetics, Inc., Sliver Spring, MD).

Histological and histomorphometrical examination. In bone metastasis experiments, mice were sacrificed at day 28 and the hindlimbs were dissected, fixed with 10% neutral buffered formalin for 48 h, decalcified in 4.13% EDTA (pH 7.4) for 14 days. In lung metastasis experiments, mice were sacrificed at day 49 and the lungs were dissected and fixed with 10% neutral buffered formalin for 48 h. All specimens were embedded in paraffin following the conventional method. The paraffin sections were stained with hematoxylin and eosin (H&E) or tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts (16).

Tumor burden in bone. Histomorphometrical analysis of the metastatic tumor burden in the hindlimbs was performed as described previously (16).

Osteoclast number. The number of TRAP-positive multinucleated osteoclasts at the tumor-bone interface was counted in 5 fields of each section at magnification x400 and expressed

per millimeter of the interface distance as described previously (16).

Tumor burden in lung. The number of metastatic foci in the lungs was counted under a dissecting microscope. Tumor area was quantified using histological sections by measuring the total tissue area per lung section (D1) and metastasis present in the same area (D2) using Image Pro Plus. Tumor area (%) was calculated by the ratio D2:D1 as described previously (17).

Immunohistochemistry. Antigen retrieval was performed by incubating the sections in citrate buffer at 95˚C for 30 min. Immunohistochemical staining was performed using a mouse monoclonal anti-cadherin-11 antibody (dilution 1:100) and VectaStain ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's protocol. Chromogen was developed using DAB substrate kit (Vector Laboratories).

Homing assay. Twelve hours before cell inoculation, the MDA-MB-231 clones were labeled with Vybrant CFDA-SE Cell Tracer Kit. The labeled cells ($1x10^6$) were inoculated into the left cardiac ventricle of 6-week-old athymic nude mice. Forty-eight hours later, bone marrow cells of the hindlimbs were flushed out and the number of the labeled cells was counted using a fluorescence-activated cell sorter (FACS; FACSort, BD Biosciences, San Jose, CA).

Cell migration assay. A layered culture system was developed using the technique described previously (18). Briefly, NIH3T3-3 or MC3T3-E1 cells ($5x10^4$ cells) were plated onto inverted transwell polycarbonate membranes (polyester membranes 3422; Corning Costar Co., Cambridge, MA). After the cells adhered firmly to the membranes, the inserts were turned bottom side up and placed into 24-well plates. The MDA-MB-231 clones ($5x10^4$ cells) labeled with Vybrant CFDA-SE Cell Tracer Kit were added into each insert well. After 8-h incubation, tumor cell migration was defined as the mean cell number of fluorescently-labeled migrated cancer cells in 5 microscopic fields at magnification x400.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from 8-h co-cultures of the MDA-MB-231 clones with NIH3T3-3 or MC3T3-E1 using RNeasy kit (Qiagen K.K., Tokyo, Japan) and treated with DNase (Wako Pure Chemical Industries) for 30 min at 37˚C. After denaturation of total RNA at 70˚C for 10 min, cDNA was synthesized with oligo-dT primer (Promega K.K., Tokyo, Japan) and PowerScript reverse transcriptase (BD Biosciences). PCR amplification was performed by using the following specific primers and cycling parameters; human PTHrP (products: 534 bp): forward primer, 5'-CAAGATTTACGGC GACGATT-3'; reverse primer, 5'-GGGCTTGCCTTTCTTTT TCT-3', 30 sec at 94˚C, 45 sec at 57˚C, 45 sec at 72˚C for 30 cycles; human GAPDH (products: 415 bp): forward primer, 5'-CATGGAGGAGGCTGGGGCTC-3'; reverse primer, 5'-CACTGACACGTTGGCAGTGG-3', 30 sec at 94˚C, 45 sec at 55˚C, 45 sec at 72˚C for 25 cycles. PCR products were separated by 2% agarose gel electrophoresis, and stained with ethidium bromide. The size of the fragments was confirmed by reference to 100-bp DNA ladder. Quantification of amplified mRNA was done by densitometry assisted by the Image analysis software (Scion Image, Scion Corporation, Frederick, MD). The results were expressed relative to the control and normalized to GAPDH.

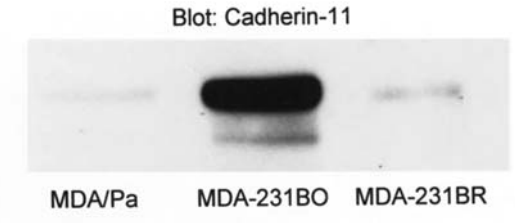

Figure 1. Cadherin-11 expression in the parental and the bone and brain-seeking clones of MDA-MB-231 cells (MDA/Pa, MDA-231BO and MDA-231BR, respectively). Expression of the intact form of cadherin-11 was determined by Western blotting.

Osteoclast-like cell formation

Conditioned medium (CM). The MDA-MB-231 clones were co-cultured with NIH3T3-3 or MC3T3-E1 cells ($5x10^5$ cells each) in 10-cm dish for 48 h. The CM was harvested and stored at -20˚C until use.

Bone marrow cultures. Mouse bone marrow cells were obtained from 4-week-old male ddY mice (Japan SLC) as described previously (17). The cells were incubated in αMEM supplemented with 10% FBS for 2 h and non-adherent cells containing hemopoietic osteoclast precursors and stromal cells were harvested. The collected marrow cells ($1x10^6$ cells/well) were cultured in the presence of PTH-rP (50 ng/ml, Sigma-Aldrich) for 6 days. On days 2 and 4, the fresh culture media (100 μl) containing PTH-rP was gently added to each well. On day 6, the cells were fixed and stained for TRAP using a commercial kit (Sigma-Aldrich). TRAP-positive multi-nucleated cells with >3 nuclei were defined as osteoclast-like cells and manually counted under a microscope. Some wells received the CM (100 μl) harvested from the co-cultures with or without 80 μg/ml anti-PTH-rP antibody on days 0, 2 and 4.

Statistical analysis. The data were analyzed by One-way ANOVA followed by Fisher's PLSD *post hoc* test (StatView; SAS Institute Inc., Cary, NC) for determination of differences between more than two groups. Student's t-test or Mann-Whitney U test was conducted when two groups were compared. P-values of <0.05 were considered statistically significant. The data are presented as mean ± SE.

Results

Expression of cadherin-11 in bone-seeking cancer cells. To investigate whether cadherin-11 expression is associated with the bone-seeking nature of breast cancer cells, we firstly examined the expression of cadherin-11 in MDA/Pa cells and bone- (MDA-231BO) and brain-seeking clones (MDA-231BR) that were established in our laboratory (15). Western blot analysis demonstrated that the cadherin-11 expression in MDA-231BO was markedly increased compared with MDA/Pa and MDA-231BR (Fig. 1).

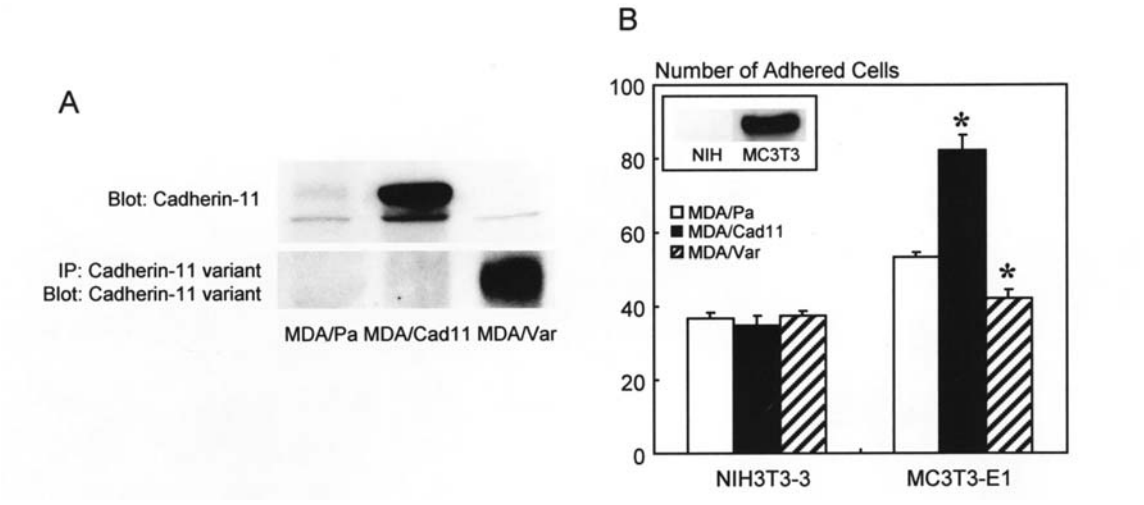

Figure 2. Establishment of MDA-MB-231 overexpressing cadherin-11. (A) Expression of the intact (upper) and the variant (lower) forms of cadherin-11 in MDA/Pa, MDA/Cad11 and MDA/Var. (B) Attachment of the MDA-MB-231 clones to NIH3T3-3 or MC3T3-E1 cells. Expression of cadherin-11 in NIH3T3-3 (NIH) and MC3T3-E1 (MC3T3) is shown in the inset. Data are expressed as number of cancer cells adhered. Data are mean ± SE (n=4/group). *Significantly different from MDA/Pa adhered to MC3T3-E1 cells ($p<0.05$).

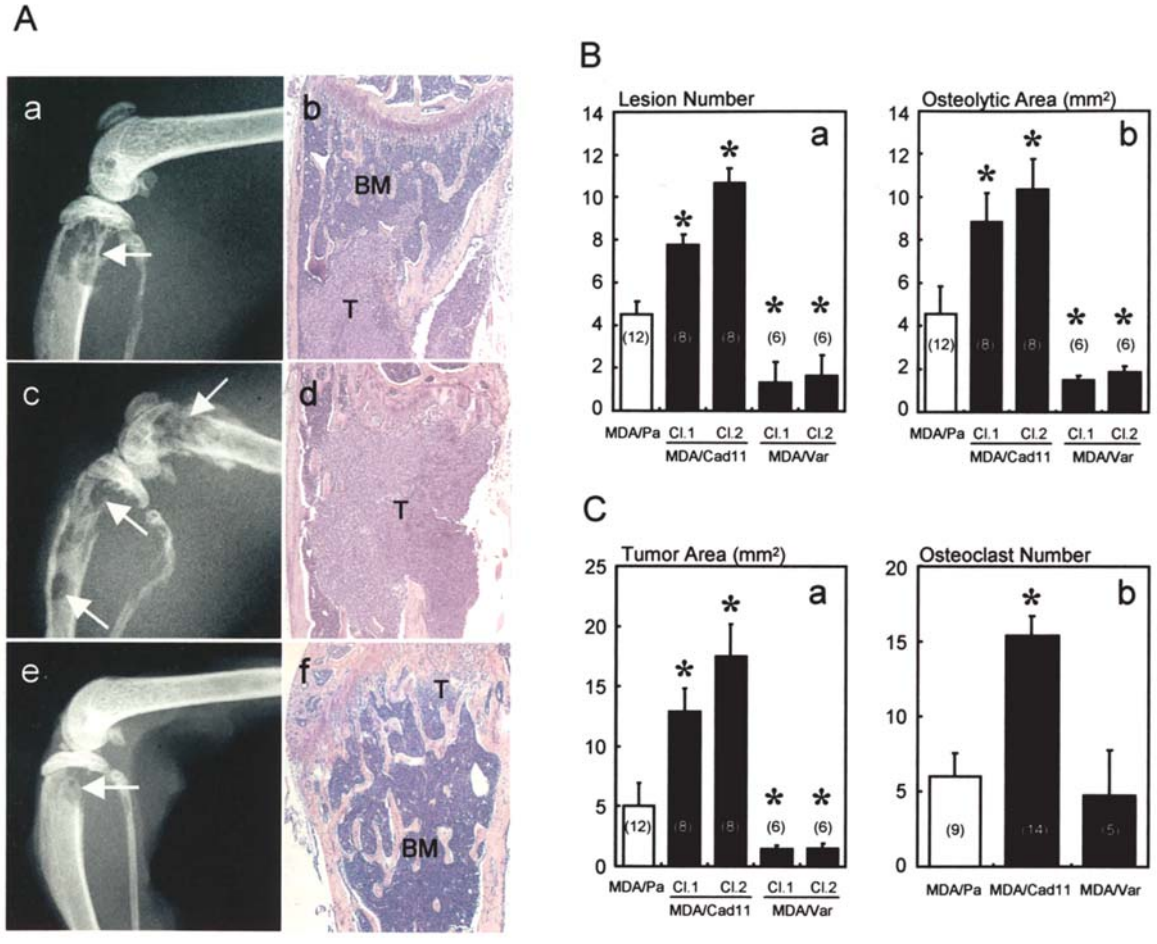

Figure 3. Bone metastases of MDA-MB-231 overexpressing cadherin-11. (A) Representative radiological and histological view of bone metastases of MDA/Pa (a and b), MDA/Cad11 (c and d) and MDA/Var (e and f) in the hindlimbs at day 28. Arrows indicate osteolytic lesions. Histological sections were stained with H&E. (T, tumor; BM, bone marrow; original magnification x50). (B) Radiographical analysis of the number (a) and the area (b) of osteolytic lesions of MDA/Pa, MDA/Cad11 and MDA/Var. Two clones (Cl.1 and 2) of MDA/Cad11 and MDA/Var were tested in this study. Data are expressed as number or area (mm^2) of osteolytic lesions per mouse. Numbers in parentheses indicate the number of mice studied (combination of two separate experiments). Data are mean ± SE. *Significantly different from MDA/Pa ($p<0.05$). (C) Histomorphometrical examination of tumor burden and osteoclast number in bone metastases of MDA/Pa, MDA/Cad11 and MDA/Var. Data are expressed as tumor area (mm^2) per mouse (a) or osteoclast number per mm tumor-bone interface (b). Numbers in parentheses indicate the number of mice studied (combination of two separate experiments). Data are mean ± SE. *Significantly different from MDA/Pa ($p<0.05$).

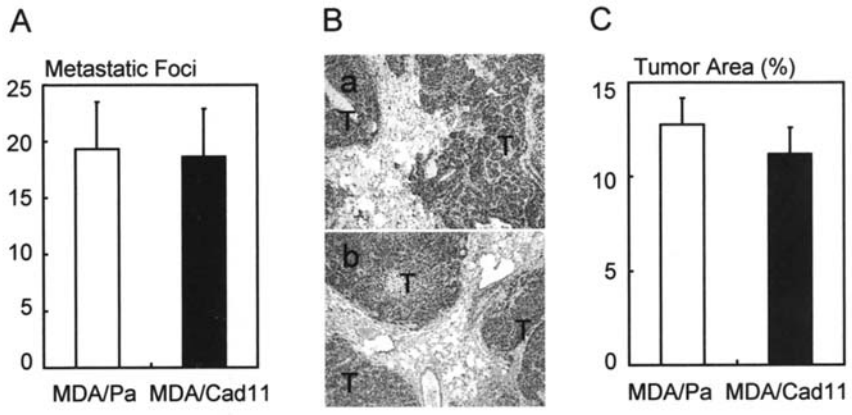

Figure 4. Lung metastases of MDA-MB-231 cells overexpressing cadherin-11. (A and C) The number (A) and the area (C) of metastases in lung were determined as described in Materials and methods. Data are expressed as number of metastatic foci per mouse (A) or tumor area (%) (C). Data are shown as mean ± SE (n=8/group). (B) Representative histological view of lung metastases of MDA/Pa (a) and MDA/Cad11. (H&E staining; T, tumor; original magnification x100).

Effects of cadherin-11 overexpression on bone metastases. To examine the role of cadherin-11 in the development of bone metastases, we established MDA-MB-231 cells stably transfected with the intact and the variant forms of cadherin-11 cDNA (MDA/Cad11 and MDA/Var, respectively) (Fig. 2A). Cell proliferation of these transfectants in monolayer cultures was not different from MDA/Pa (data not shown). Cell dissociation assay that indicates the Ca^{2+}-dependent cell adhesion ability showed that MDA/Cad11 acquired higher adhesiveness than MDA/Pa and MDA/Var (data not shown). Furthermore, MDA/Cad11 showed increased attachment to the monolayer of MC3T3-E1 cells that inherently expressed cadherin-11 (11) compared with MDA/Pa and MDA/Var (Fig. 2B), whereas there were no differences in the attachment to the monolayer of cadherin-11-negative NIH3T3-3 cells among MDA/Pa, MDA/Cad11 and MDA/Var (Fig. 2B). These results suggest that the wild-type and variant cadherin-11 introduced in MDA/Pa cells were biologically functional as we expected. We then examined the capacity of these transfectants to develop bone metastases following intracardiac inoculation into female nude mice. Radiographical analyses demonstrated that the number and size of osteolytic lesions were significantly increased in MDA/Cad11 compared to MDA/Pa (Fig. 3A and B). In contrast, MDA/Var developed reduced osteolytic bone metastases (Fig. 3A and B). Histological examination also showed that the metastatic tumor burden in bone was increased in MDA/Cad11, while it was significantly decreased in MDA/Var (Fig. 3A and C). The number of TRAP-positive osteoclasts at tumor-bone interface was significantly increased in the bone metastases of MDA/Cad11 compared with MDA/Pa and MDA/Var (Fig. 3C).

Role of cadherin-11 in lung metastases. To study the role of cadherin-11 in metastasis to non-bone sites, we examined lung metastases of MDA/Cad11 by tail vein inoculation. Macroscopic and histological examinations showed that the number of metastatic foci and the tumor burden in lung was not changed in MDA/Cad11 compared with MDA/Pa (Fig. 4). These results suggest that cadherin-11 selectively promotes bone metastases.

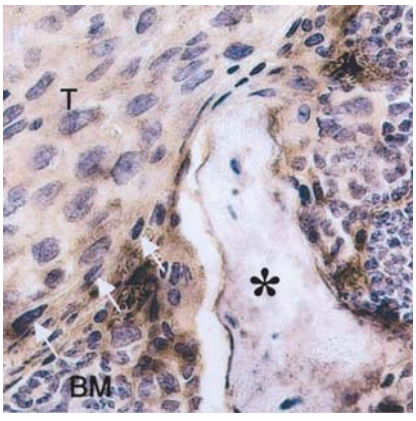

Figure 5. Immunohistochemical examinations of bone metastases of MDA/Cad11 at day 28. Cadherin-11-positive tumor cells (T) are in direct contact with cadherin-11-positive bone marrow stromal cells (arrows). (BM, bone marrow; asterisk, bone; original magnification x400).

MDA/Cad11 cell arrest in bone marrow. Immunohistochemical examination revealed that MDA/Cad11 cells were in direct contact with cadherin-11-positive spindle-shaped stromal cells in bone (Fig. 5), showing that metastatic MDA/Cad11 cells establish a homophilic cell-cell interactions with residing bone marrow stromal cells.

To determine the mechanism by which cadherin-11 selectively increased bone metastases, we examined whether cadherin-11 increases cancer cell arrest in bone marrow *in vivo*. To approach this, fluorescent dye-labeled MDA/Pa, MDA/Cad11 and MDA/Var cells were inoculated into the heart and the number of arrested cells in bone marrow was counted by FACS 48 h later. The number of MDA/Cad11 arrested in bone marrow was significantly greater than MDA/Pa (Fig. 6). In contrast, the arrest of MDA/Var was significantly decreased (Fig. 6).

Effects of cadherin-11 on cell migration. We next examined the effects of cadherin-11 on cell migration using transwell chambers as described in Materials and methods (18). Migration of MDA/Cad11 cells was significantly stimulated when cultured with MC3T3-E1 cells that expressed

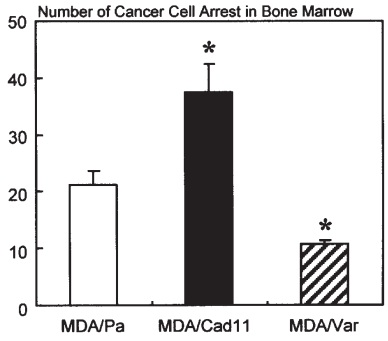

Figure 6. Early stage colonization of the MDA-MB-231 clones in bone marrow. Forty-eight hours after the intracardiac injection, the number of cancer cells arrested in bone marrow was determined by FACS as described in Materials and methods. Data are expressed as number of cancer cells arrested in bone per mouse. Data are mean ± SE (n=10/group). *Significantly different from MDA/Pa ($p<0.05$).

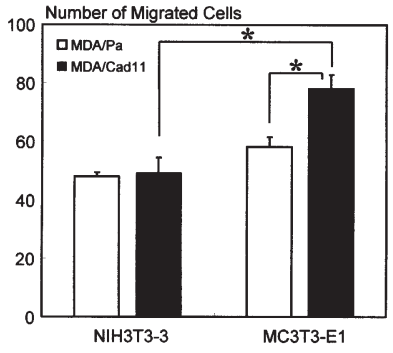

Figure 7. Effects of cadherin-11 on cell migration. MDA/Pa and MDA/Cad11 cells were labeled with fluorescent dye and the number of cells migrated to the opposite side of the membranes was counted. Data are expressed as migrated cell number per mm^2 membrane area. Data are mean ± SE (n=4/group). *Significantly different ($p<0.05$).

cadherin-11 (Fig. 7). In contrast, there was no significant difference in the number of migrated cells between MDA/Pa and MDA/Cad11 cultured with NIH3T3-3 cells that did not express cadherin-11 (Fig. 7).

Effects of cadherin-11-mediated cell adhesion on osteoclast formation. To determine the mechanism of increased osteoclast number in the bone metastases of MDA/Cad11 (Fig. 3C), we examined the production of osteoclast-stimulating factors in the MDA-MB-231 clones as a consequence of the homophilic interactions with bone marrow stromal/osteoblastic cells via cadherin-11. Semi-quantitative RT-PCR analysis demonstrated that mRNA expression of PTH-rP, a major osteoclastogenic factor produced by MDA-MB-231 cells (19), was up-regulated in the co-cultures of MDA/Cad11 and MC3T3-E1 cells but not of MDA/Cad11 and NIH3T3-3 cells (Fig. 8A). Since we used human-specific primers in these experiments, the results shown represent PTH-rP mRNA expression in the MDA-MB-231 clones of human origin. There was no up-regulation of PTH-rP mRNA expression in the co-cultures of MDA/Pa or MDA/Var and MC3T3-E1 (Fig. 8A).

The CM from the co-cultures of MDA/Cad11 and MC3T3-E1 markedly increased TRAP-positive osteoclast-like cell formation (Fig. 8B). This stimulatory effect was blocked by a neutralizing antibody to PTH-rP (Fig. 8B). The CM harvested from the co-cultures of other combinations also showed stimulatory effects presumably due to intrinsic PTH-rP production by MDA-MB-231 cells but to a significantly lesser extent (Fig. 8B).

Discussion

Cancer metastasizes to distant organs with distinct organ preference (1-3). Since Paget proposed 'Seed and Soil'

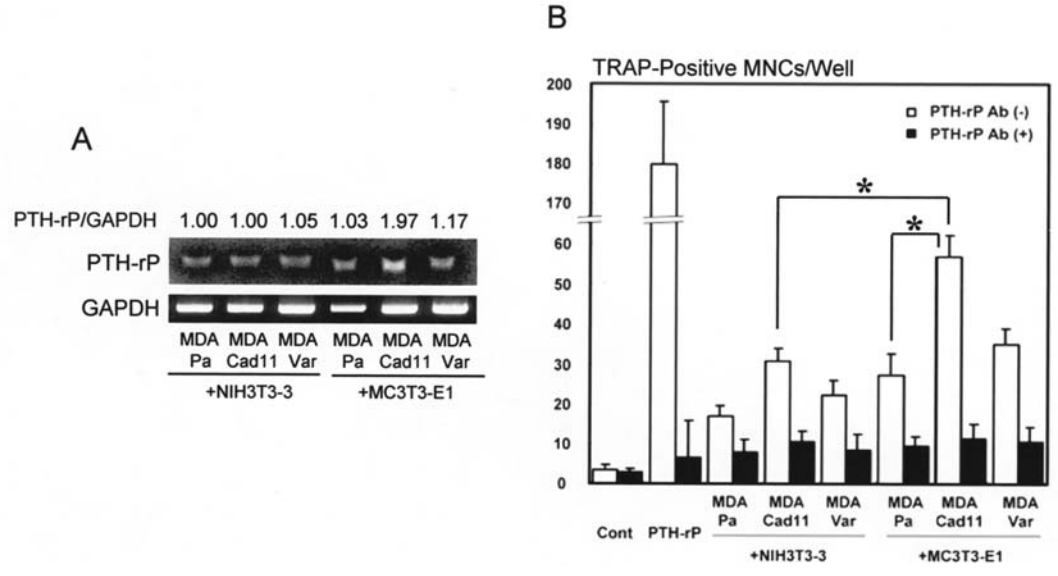

Figure 8. Production of osteoclastogenic activity in the co-cultures of the MDA-MB-231 clones with osteoblastic cells. (A) Semi-quantitative RT-PCR analysis of PTH-rP mRNA expression in the co-cultures of MDA-MB-231 clones with NIH3T3-3 or MC3T3-E1 cells. Since the primer sets used in this study are human specific, the data shown here indicate mRNA expression of human PTH-rP and GAPDH in MDA/Pa, MDA/Cad11 or MDA/Var that are of human origin. Quantification of amplified mRNA was done as described in Materials and methods and indicated as fold induction to the control and normalized to GAPDH. (B) Osteoclast-like cell formation in mouse bone marrow cells cultured with the CM obtained from the co-cultures of MDA/Pa, MDA/Cad11 or MDA/Var and MC3T3-E1 or NIH3T3-3 cells in the absence (open bar) or presence (closed bar) of a neutralizing antibody to human PTH-rP. Data are expressed as number of TRAP-positive multinucleated cells (MNCs) per well per 48-well plate. Data are mean ± SE (n=4/group). *Significantly different ($p<0.05$).

theory more than a century ago (20), the interactions between metastatic tumor cells and host environment have been implicated in organ-selective metastasis. Recent studies using microarray analyses of the bone- (21) and lung-seeking clones (22) of MDA-MB-231 cells demonstrated that the gene expression profile of these clones was distinct from the parental cells. Identification of these signatures of bone and lung metastases has increased our understanding of the mechanism underlying organ-selective metastasis of breast cancer. However, key molecules responsible for organ-selective metastasis still need to be elucidated.

Interactions of cancer cells with organ environments mediated by CAMs have been thought to contribute to organ preferential metastasis (4-6). Cadherins have been implicated in cancer invasion and metastasis (9,10). We previously reported that the classical type 1 cadherin family member E-cadherin inhibited breast cancer metastasis to bone in a well-characterized animal model of bone metastasis (23). Consistent with our results, E-cadherin has been shown to be a tumor suppressor (9,10). In contrast, cadherin-11 has been proposed to be a tumor promoter (12,24). Earlier studies showed that aggressive human breast tumor cells express elevated levels of cadherin-11 (12). Along this line, it is notable that stromal and osteoblastic cells in bone marrow inherently express cadherin-11 as well (11). These observations collectively led us to hypothesize that cadherin-11 is involved in the preferential metastasis of breast cancer cells to bone via homophilic interactions with resident bone marrow stromal/osteoblastic cells.

In the present study, we showed that the cadherin-11 expression in the bone-seeking clone of MDA-MB-231 cells (15) was markedly increased compared to the parental and the brain-seeking clone, suggesting an involvement of cadherin-11 in the bone-seeking nature of breast cancer. This notion is supported by the finding that the introduction of the intact form of cadherin-11 enhanced the development of bone metastases of MDA-MB-231 cells. Importantly, however, cadherin-11 overexpression in MDA-MB-231 cells did not change the metastasis to lung. These results suggest that cadherin-11 specifically promotes bone metastases of breast cancer.

Of note, overexpression of the variant form of cadherin-11 reduced the cell adhesion and the development of bone metastases. It has been reported that the splice variant of cadherin-11 shows no homophilic cell-cell adhesion property. However, it has not been defined whether this variant form acts to inhibit the function of intact cadherin-11. Kashima *et al* suggested that the secreted form of cadherin-11, which is derived from the intact form by proteinase cleavage, impedes the function of the intact form in osteosarcoma cells (14). Our results show that the variant form suppresses bone metastases. These results are consistent with the notion that the establishment of homophilic interaction between metastatic cancer cells and bone marrow stromal cells via cadherin-11 is critical to the development of bone metastases.

Cancer cell arrest in target organs is a critical step for the initiation of metastases (2). Our experiments using FACS analysis of the fluorescent dye-labeled MDA-MB-231 cells showed that the early settlement of cancer cells following heart inoculation was significantly increased in MDA/Cad11 compared with MDA/Pa. Furthermore, MDA/Var displayed significantly less arrest than MDA/Pa. These results suggest that cadherin-11 plays an important role in the initial colonization of breast cancer cells in bone marrow via promoting the homophilic cell-cell interactions with resident stromal/osteoblastic cells. Increased cancer cell arrest in bone marrow consequently leads to increased bone metastases.

Directed cell migration is also crucial for cancer cells to metastasize to distant organs. It has been shown that N-cadherin increases cell migration and metastases (25). Our study using the Boyden chamber assay showed that the directed cell migration of MDA/Cad11 was promoted when they interacted with cadherin-11-expressing MC3T3-E1 cells, whereas the interactions with non-cadherin-11-expressing NIH3T3-3 cells did not stimulate MDA/Cad11 migration. These results suggest that cadherin-11 also plays a critical role in directed migration of breast cancer cells through the homophilic cell-cell interactions.

The establishment of distant metastases in target organs requires not only migration and homing of cancer cells but also successful proliferation and survival in the local environment (2,3). A key essential event for metastatic cancer cells to colonize bone is osteoclastic bone resorption. Bone resorption releases a variety of bone-stored growth factors such as IGFs and TGFß into the bone microenvironment, which in turn promotes cancer cell proliferation, survival and metabolism (2,3,26). In this context, it should be noted that our histological examination demonstrated that osteoclastic bone resorption was significantly increased in the bone metastases of MDA/Cad11 compared to MDA/Pa and MDA/Var. Consistent with these results, the co-cultures of MDA/Cad11 and MC3T3-E1, both of which express cadherin-11, increased the mRNA expression of PTH-rP, a well-described potent osteoclastogenic cytokine, in MDA/Cad11 cells. Furthermore, the CM harvested from the co-cultures of MDA/Cad11 and MC3T3-E1 stimulated TRAP-positive osteoclast-like cell formation in mouse bone marrow cultures and this stimulatory effect was blocked by a neutralizing antibody to human PTH-rP. These results suggest that the contact with cadherin-11 expressed in bone marrow stromal/osteoblastic cells stimulates the production of PTH-rP by MDA/Cad11, leading to increased osteoclastic bone destruction and bone metastases. Although the mechanism by which the homophilic cell-cell interactions via cadherin-11 increase PTH-rP production in breast cancer cells is currently unknown, we have previously shown that the cell-cell contact between bone marrow stromal cells and myeloma cells via vascular cell adhesion molecule 1 and α4ß1 integrin also enhanced the production of osteoclast-stimulating activity (27). The activation of cadherin signaling pathways likely plays a role in the up-regulation of PTH-rP production in MDA-MB-231 cells (28).

In conclusion, our results suggest that the expression of cadherin-11 in breast cancer cells promotes homing, directed cell migration and osteoclastogenesis via homophilic interactions with cadherin-11 expressed in resident bone marrow stromal/osteoblastic cells and thereby stimulates colonization of breast cancer cells in bone, resulting in an enhancement of bone-selective metastases. Thus, cadherin-11 is, at least in part, responsible for the preferential spread of breast cancer

cells to bone. The results also suggest that cadherin-11 is a potential therapeutic molecular target for specific and effective prevention and treatment of bone metastases.

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