

## Expression of Osteoprotegerin and RANK Ligand in Breast Cancer Bone Metastasis

Bone destruction is primarily mediated by osteoclastic bone resorption, and cancer cells stimulate the formation and activation of osteoclasts next to metastatic foci. Accumulating evidences indicate that receptor activator of NF- $\kappa$ B ligand (RANKL) is the ultimate extracellular mediator that stimulates osteoclast differentiation into mature osteoclasts. In contrast, osteoprotegerin (OPG) inhibits osteoclast development. In order to elucidate a mechanism for cancer-induced osteoclastogenesis, cells from a human breast cancer line, MDA-MB-231, were directly co-cultured with ST2, MC3T3-E1, or with primary mouse calvarial cells. Osteoclast-like cells and tartarate resistant acid phosphatase (TRAP) activities were then quantitated. We examined these cell lines and samples from breast cancer by RT-PCR for the expressions of OPG and RANKL mRNA. Compared to controls, co-culture of MDA-MB-231 cells with stromal or osteoblastic cells induced an increase in number of osteoclasts and TRAP activities. MDA-MB-231 cells alone or breast cancer samples did not express RANKL mRNA. However, co-culture of these cancer cells with stromal or osteoblastic cells induced RANKL mRNA expression and decreased OPG mRNA expression. These experiments demonstrate that direct interactions between breast cancer and stromal or osteoblastic cells induce osteoclastogenesis in vitro through modulating RANKL expression.

Key Words : *Breast Neoplasms; Bone and Bones; Neoplasm Metastasis; Osteoclasts*

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## INTRODUCTION

It has long been recognized that breast cancers can invade and grow as metastases in bone (1). Some breast cancer cell lines induce osteolytic lesions in animal models that mimic the metastatic process in clinical breast cancer (2, 3), but the factors favoring the growth of breast cancer in bone remain to be resolved. Bone destruction is primarily mediated by osteoclastic bone resorption (4), and cancer cells that have metastasized to bone stimulate the formation and activation of osteoclasts next to metastatic foci (5). Recent research has identified several factors inducing osteoclast formation (4). If different types of metastatic cancer follow the same mechanism to cause osteolytic lesions, we may focus on this target to control and prevent the damage to bone.

Accumulating evidences indicate that receptor activator of NF- $\kappa$ B ligand (RANKL), also known as osteoclast differentiation factor, is the ultimate extracellular mediator that acts on osteoclast precursors to differentiate them into mature osteoclasts in the presence of macrophage-colony stimulating factor (M-CSF) (6, 7), and that many bone resorbing factors up-regulate RANKL expression in bone marrow stromal and osteoblastic cells (6, 8). Osteoprotegerin (OPG), also known

as osteoclastogenesis inhibitory factor, inhibits osteoclast development (9, 10) because it is a decoy receptor for RANKL to block its actions (6, 7). Thus, divergent mechanisms for bone resorption may converge to the regulation of RANKL activities. Cancer cells induce formation of osteoclasts by secreting osteotropic cytokines, such as PTH-related protein (PTHrP) (11), interleukin-11 (IL-11) (12) and leukemia inhibitory factor (LIF) (13), or by direct contact with bone marrow cells (1). The molecular mechanisms for the latter are yet unclear, although osteotropic factors secreted by cancer cells could induce RANKL. In order to reveal a mechanism for cancer-induced osteoclastogenesis, we examined RANKL and OPG expression in co-cultures of bone marrow stromal or osteoblastic cells with breast cancer cells inducing osteoclast-like cell formation in vitro.

## MATERIALS AND METHODS

### Tissue specimens and animals

Thirty tissue specimens from breast cancer were obtained from thirty patients by surgical resection in KangDong Sacred

Heart Hospital, Hallym University Medical Center (Seoul, Korea). Tissue specimens were snap-frozen in liquid N<sub>2</sub> and stored at -70°C until used. Male C57 black mice (6 weeks old) were obtained from Asan Institute for Life Science (Seoul, Korea). All procedures involving animals were approved by the institutional animal care committee.

#### Isolation and culture of neonatal mouse calvarial cells

Calvariae from 3 to 5-day-old neonatal C57 black mice were dissected and cut into small pieces. Following serial treatments with 0.1% collagenase (Sigma, St. Louis, MO, U.S.A.) and 0.2% protease (Sigma, St. Louis, MO, U.S.A.), cells were collected from the supernatant (fractions 2 to 5). Cells were cultured in  $\alpha$ -MEM (minimum essential media, GIBCO, Gaithersburg, MD, U.S.A.) supplemented with 10% FCS (fetal calf serum, GIBCO, Gaithersburg, MD, U.S.A.), 100 IU/ $\mu$ L penicillin and 100  $\mu$ g/ $\mu$ L streptomycin (GIBCO, Gaithersburg, MD, U.S.A.), at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. In addition, mouse osteoblastic cell line MC3T3-E1 and bone marrow-derived stromal cell line ST2 were used as osteoblastic and stromal cells.

#### MDA-MB-231: a human breast cancer cell line

The MDA-MB-231 cell line was provided by the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI (GIBCO, Gaithersburg, MD, U.S.A.) supplemented with 10% FCS, 100 IU/ $\mu$ L penicillin, and 100  $\mu$ g/ $\mu$ L streptomycin (Life Technologies, Inc.) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. MDA-MB-231 cells were studied in co-culture systems with mouse bone marrow stromal or osteoblastic cells. As described previously (12), 2  $\times$  10<sup>5</sup> cancer cells were inoculated onto a 60 mm culture dish at 2 hr before seeding with bone marrow stromal or osteoblastic cells, prepared as described below. Serum-free conditioned media collected from the cultures after a 5-day incubation were kept at -20°C until required. Aliquots of conditioned media were diluted with  $\alpha$ -MEM supplemented with 10% FCS and used at dilutions of 1:2 and 1:5.

#### Mouse bone marrow culture for osteoclast formation

Bone marrow cells of 6-week-old male C57 black mice were obtained from femora and tibiae, and were cultured as previously described (14). Non-adherent marrow mononuclear cells were prepared and then re-suspended in  $\alpha$ -MEM supplemented with 10% FCS at 10<sup>6</sup> cells/ $\mu$ L. Cells were plated in 96 well plates (1  $\times$  10<sup>6</sup> cells/well) to test the effects of conditioned media on the formation of osteoclasts. Cultures were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C for 8 days. Cultures were fed once in every other day by removing half of the medium and replacing it with an equal volume of fresh medium.

#### Tartrate-resistant acid phosphatase (TRAP) staining

On the eighth day of bone marrow culture, cells were fixed in 10% formalin neutral buffer solution for 10 min and stained with an Acid Phosphatase Leukocyte Kit (Sigma Diagnostics, St. Louis, MO, U.S.A.) for 1 hr at 37°C. TRAP-positive multinucleated cells with more than three nuclei per well were counted.

#### TRAP activity

For TRAP activities, 100  $\mu$ L of culture media were mixed with 100  $\mu$ L of 0.2 M sodium acetate buffer, 16  $\mu$ L of 0.1 M pNPP, and 4  $\mu$ L of 2.0 M L(+) tartarate for 1 hr at 37°C. We added 16  $\mu$ L of 0.5 M NaOH and measured the optical density at 405 nm, using an ELISA plate reader.

#### RT-PCR for OPG and RANKL mRNA expression

Adherent bone marrow cells (2  $\times$  10<sup>5</sup>/60 mm dish) were co-cultured with or without cancer cells (2  $\times$  10<sup>5</sup>/60 mm dish) as described above. After 5 days, total RNAs were extracted by using RNeasy Kits (Qiagen, Santa Clarita, CA, U.S.A.) and then reversely transcribed according to the instructions of the manufacturer. Total RNAs from thirty tissue specimens of breast cancer were also extracted by using the same kits. Primer sequences (Bioneer, Korea): OPG, 5'-AACCCAGAGCGAA-ACAC-3' (sense), 5'-AAGAAGGCCTCTTCACAC-3' (antisense), RANKL, 5'-GGTCGGGCAATTCTGAATT-3' (sense), and 5'-GGGGAATTACAAAGTGC-3' (antisense). PCR was performed on a 9600 thermal cycler (Perkin Elmer, Norwalk, CT, U.S.A.), at the following conditions: at 94°C for 1 min (1 cycle), at 55°C for 40 sec, and at 72°C for 1 min (32 cycles) for OPG; at 94°C for 1 min (1 cycle), at 62°C for 1 min, and at 72°C for 1 min (32 cycles) for RANKL. For semi-quantitative RT-PCR, one  $\mu$ g of total RNA was treated with DNase I, reversely transcribed, and subjected to PCR for OPG and RANKL. Mouse  $\beta$ -actin mRNA expression was also examined by RT-PCR as an internal control.

#### Statistical analysis

All the experiments were repeated at least three times. Data were expressed as mean  $\pm$  SE and analyzed by one-way analysis of variance. An unadjusted *p* value of less than 0.05 was considered to be significant.

## RESULTS

#### Cancer cells adhered to bone marrow cells induce formation of TRAP-positive multinucleated cells

Direct co-cultures of mouse bone marrow cells with MDA-

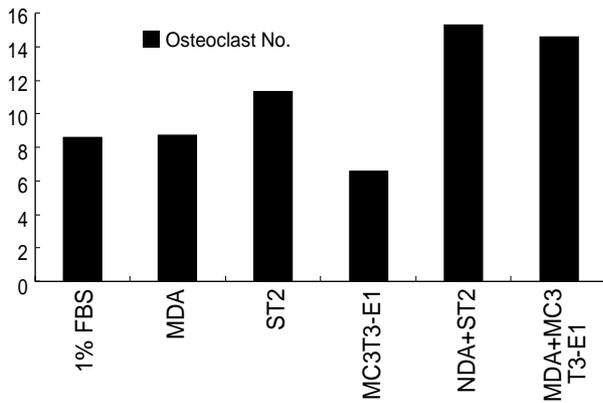


Fig. 1. Effects of conditioned media on osteoclast generation in bone marrow cultures. Conditioned media from co-culture of MDA-231 with ST2 or MC3T3-E1 cells increase TRAP-positive multinucleated cell formation, compared to controls.

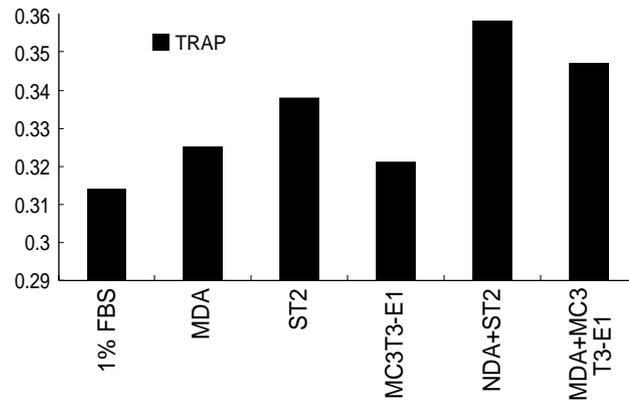


Fig. 2. Effects of conditioned media on TRAP activity in bone marrow cultures. Conditioned media from co-culture of MDA-231 with ST2 or MC3T3-E1 cells increase TRAP activity, compared to controls.

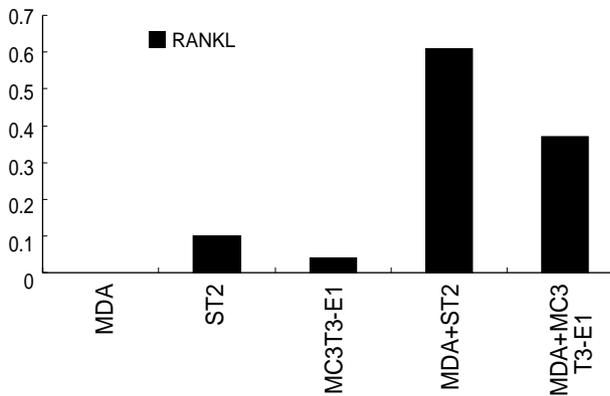


Fig. 3. Expression of RANKL mRNA assessed by RT-PCR. The results were shown as a ratio of RANKL to actin mRNA. Co-culture of MDA-231 with ST2 or MC3T3-E1 cells induces expression of RANKL mRNA.

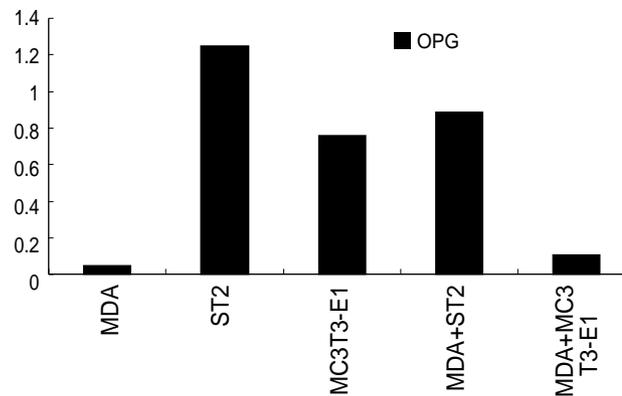


Fig. 4. Expression of OPG mRNA assessed by RT-PCR. Co-culture of MDA-231 with ST2 or MC3T3-E1 cells inhibit expression of OPG mRNA.

MDA-231 cells, without osteotropic agents, generated some TRAP-positive multinucleated cells.

**MDA-MB-231 conditioned medium increases the formation of osteoclasts in mouse bone marrow cultures**

To test the effects of the factor (s) released from the tumor cells on the formation of osteoclasts, bone marrow cells were cultured either in the presence or the absence of MDA-MB-231 conditioned medium from co-culture with osteoblastic (MC3T3-E1) or stromal (ST-2) cells. The use of conditioned medium, even at the highest dilution (1:5), significantly increased the number of TRAP (+) multinucleated cells. The mean number of TRAP (+) multinucleated cells/field was 16.4 in treated cultures, compared with 8.6 in controls (Fig. 1). TRAP activities were also significantly increased with the addition of the conditioned media from co-culture (Fig. 2).

**Co-culture of cancer cells with bone marrow cells induces RANKL expression**

In order to clarify the mechanism whereby cancer cells induce osteoclast-like, TRAP-positive multinucleated cell to form in vitro through their direct contact with bone marrow cells, we examined the expression of RANKL mRNA by RT-PCR. Cancer cell lines or tissue specimens from breast cancer did not express RANKL mRNA. Co-cultures of MDA-MB-231 with osteoblastic (MC3T3-E1) or stromal (ST-2) cells induced the RANKL mRNA expression (Fig. 3).

**Co-culture of cancer cells with bone marrow cells reduces OPG expression**

Osteoblastic (MC3T3-E1) or stromal (ST-2) cells alone expressed OPG. Co-culture of MDA-MB-231 cells with osteoblastic (MC3T3-E1) or stromal (ST-2) cells reduced the expression of OPG. Cancer cells alone barely expressed OPG (Fig. 4).

## DISCUSSION

Breast cancers commonly cause osteolytic metastases in bone, a process that depends upon osteoclast-mediated bone resorption, but the mechanism of osteoclast activation is not yet clear. In the present study we assessed whether a well-known human breast cancer cell line (MDA-MB-231) could cause osteoclasts to form in mouse bone marrow cultures. This cell line increases osteoclast formation *in vitro* and forms osteolytic bone metastases in mice (6). We demonstrated that in bone marrow cultures, the conditioned medium harvested from MDA-MB-231 increased the formation of multinucleated TRAP-positive cells. These data suggest that MDA-MB-231 produces factors that increase the formation of osteoclasts in bone marrow culture.

Teti *et al.* (4) observed that melanomas as well as breast carcinomas could induce osteoclast formation *in vitro*. Experiments where bone marrow cells contacted tumor cells directly showed similar results to those where bone marrow cells were incubated in serum-free media conditioned by tumor cells. This effect was dose-dependent and indicated that cell-cell contact was not required. Furthermore, similar effects were observed whether the conditioned media were added to the bone marrow cultures during the first 1-6 days or the last 6-10 days of osteoclast formation. Ono *et al.* (12) also examined mechanisms by which the breast cancer cell line, BALB/c-MC, induces osteoclast formation. BALB/c-MC stimulated osteoclast formation through direct contact with bone marrow cells, and involved PGE<sub>2</sub> released from bone marrow cells. Co-culture of either mouse melanoma B16 cells or breast cancer BALB/c-MC cells with mouse bone marrow cells induced osteoclast-like cells, which were not observed when the cancer cells were segregated from the bone marrow cells (10). Thus, some tumor types seem to require direct contact with marrow cells to induce osteoclast formation, whereas other tumor types do not require direct contact.

When bone-seeking cancer cells stimulate osteoclast formation, the process involves several humoral factors, such as PTHrP (2), IL-11 (15), LIF (16), and PGE<sub>2</sub> (12). However, since the list was compiled from studies of several types of cancer cells, these factors may not be universally used. Central to the process is a group of TNF receptor and TNF ligand family members. RANKL, also known as ODF, OPGL or TRANCE, promotes osteoclast formation by interacting with the receptor RANK, and the process is inhibited by the decoy receptor, OPG (7, 17-22). It is now considered as a direct mediator of many osteotropic factors (5). Our experiment revealed that neither bone marrow stromal cells nor breast cancer cells alone expressed RANKL mRNA. However, co-culture of these cancer cells with bone marrow stromal cells, or with osteoblastic cells, induced RANKL expression. Moreover, when bone marrow stromal cells were co-cultured with cancer cells, the bone marrow cells inhibited OPG. Thus, enhanced osteoclast formation in the presence of cancer cells might be due to the increase in RANKL activities. These results suggest that in

metastases where bone is damaged, interactions between cancer cells and bone marrow cells could induce RANKL and suppress OPG.

Overproduction of PTHrP by breast cancer cells may be important in determining their ability to establish and grow in bone. Although Martin (7) found that breast cancers do not produce RANKL themselves, they do produce PTHrP, and this can influence bone cells to produce RANKL and decrease OPG, thereby favoring osteoclast formation and metastasis growth. Thomas *et al.* (11) determined that the breast cancer cell lines MDA-MB-231, MCF-7, and T47D as well as primary breast cancers do not express RANKL but express OPG and RANK. Because M-CSF and RANKL are essential for osteoclast formation, Mancino *et al.* (6) hypothesized that MDA-231 cells could stimulate osteoclastogenesis by producing one or both of these cytokines themselves or by stimulating bone marrow stromal/osteoblastic cells to produce them. Soluble RANKL alone, or MDA-231 cells without added RANKL did not support osteoclast formation from hematopoietic cells. However, co-culture of MDA-231 cells with hematopoietic cells and added soluble RANKL stimulated significant osteoclast formation, indicating that MDA-231 cells produce M-CSF. In contrast to their ability to produce M-CSF, MDA-231 do not produce RANKL. However, when co-cultured with the murine bone marrow stromal cell line UAMS-33, MDA-231 cells increased their RANKL expression significantly. These results indicate that MDA-231 cells could cause osteoclasts to form, partly by secreting M-CSF, and partly by stimulating RANKL expression in host stromal/osteoblastic cells. IL-6 type cytokines may be involved in stimulating RANKL expression.

Accumulating evidences indicate that RANKL produced by bone marrow stromal cells is the final extracellular regulator of osteoclast development. OPG is a soluble decoy receptor for RANKL, and antagonizes its activities (20, 21). Thus, the ratio of RANKL to OPG could regulate osteoclastogenesis. When adherent bone marrow cells were studied, the presence of MDA-MB-231 breast cancer cells significantly decreased the concentration of OPG in culture media. This might increase the levels of free RANKL, enhancing osteoclast formation. Because RANKL acts directly on hematopoietic osteoclast precursors (20), observations shown here and by others suggest that when cancer cells induce osteoclast development, it is primarily by causing bone marrow cells to express RANKL. Therefore, RANKL and OPG may be useful targets for treating bone metastases.

To elucidate the mechanism of osteoclastogenesis and bone destruction in a mouse model of breast cancer, Kitazawa *et al.* (5) used *in situ* hybridization to study the expression of RANKL mRNA. In early stages of bone invasion, spindle-shaped mesenchymal cells and osteoblasts on the bone surface expressed RANKL. At this stage, TRAP-positive osteoclasts were already seen next to cancer cells. Cancer cells did not express RANKL but did express PTHrP. At a later stage, when cancer

cells had invaded and started to damage the bone, RANKL mRNA was detected mainly on the osteoblastic cells around the eroded bone surface. TRAP-positive osteoclasts at the same surfaces also expressed RANKL. Prior to direct invasion by the cancer cells, RANKL expression was confined to the osteoblastic cell lineage, suggesting that cancer-derived factors like PTHrP plays a central role in inducing RANKL, eventually leading to bone destruction. The activities of OPG were studied in a syngeneic tumor model of humoral hypercalcemia of malignancy. OPG blocked tumor-induced increases in bone resorption and hypercalcemia and rapidly normalized blood calcium. In tumor-bearing mice, OPG treatments reduced osteoclast activities. The potent effects of OPG in this model suggest it could be used therapeutically (13).

In conclusion, cell-cell interaction between cancer and bone marrow cells possibly stimulates osteoclast-like cell formation by inducing RANKL expression and suppressing OPG secretion. This mechanism may be ubiquitous in bone destruction in malignancy because in different tumor types, the actions converge of RANKL. In addition, characterization of this simple model of tumor-induced osteoclast generation identified possible molecular targets for treating osteolytic metastases.

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