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Human bone marrow adipocytes support dexamethasone-induced osteoclast differentiation and function through RANKL expression

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ABSTRACT

The TNF-family molecule, Receptor Activator of Nuclear factor κ B Ligand (RANKL) is known as a key regulator for bone remodeling, and is essential for the development and activation of osteoclasts. In this study, we examined the regulation of RANKL in primary human bone marrow adipocytes and the relationship between bone marrow adipocytes and bone metabolism. RANKL expression and the RANKL/osteoprotegerin (OPG) mRNA ratio in marrow adipocytes increased following dexamethasone treatment. In co-cultures of human osteoclast precursors and bone marrow adipocytes with dexamethasone, osteoclast precursors differentiated to TRAP-positive multinuclear cells. Moreover, the ability of bone resorption was confirmed in co-culture in flasks coated with calcium phosphate film. Osteoclast precursor differentiation and bone resorption were blocked by RANKL antibody pretreatment. TRAP-positive multinuclear cells did not form in co-culture without cell-to-cell contact conditions. We conclude that primary human bone marrow adipocytes have the ability to promote osteoclast differentiation and activities, similar to osteoblasts and other RANKL-expressing cells.

There are various hypotheses about the function of bone marrow adipocytes, which are present in large quantities in the choke marrow space. Some hold that bone marrow adipocytes function as 1) a spacer between hematopoietic cells and bone tissue, 2) a fat metabolism organ, 3) an energy storehouse, or 4) support cells of hematopoiesis and bone metabolism (14). In contrast, it is clear that subcutaneous and visceral fats are not only simple spacers, but also energy storehouses. Subcutaneous and visceral fats also serve as metabolic organs secreting physiologically active substances named adipokines, which are associated with hypertension and metabolic syndrome (23). The adipokines, such as leptin, adiponectin, and TNF-α, act on osteoclasts and osteoblasts that are associated with bone metabolism (1, 8, 10, 17, 22). However, their function has not yet been fully elucidated.

It is known that with aging, the composition of the bone marrow shifts to favor the presence of adipocytes, and osteoclast activity increases while osteoblast function declines, resulting in osteoporosis (14). Several reports have described the relationship between bone marrow adipocytes and bone metabolism. The BMS2 adipocyte cell line from murine bone marrow stromal cells promotes the differentiation of osteoclast-like cells in the presence of vitamin D (7). C3 protein, which is secreted during the initial stage of adipogenesis in murine bone marrow stromal cells, enhances osteoclast differentiation (15).

Receptor Activator of Nuclear factor κ B Ligand (RANKL) is an essential molecule in osteoclast differentiation, as is the decoy receptor molecule os-

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teoprotegerin (OPG). Marrow stromal cell-derived osteoblasts can support osteoclast differentiation through RANKL expression. Bone marrow adipocytes and osteoblasts differentiate from a common progenitor of marrow mesenchymal stem cells (13). A recent report showed that 3T3-L1 cells, which are a mouse embryonic fibroblast/adipose-like cell line, and rat adipose tissue, express RANKL (2).

Given these findings, we predicted that bone marrow adipocytes stimulate osteoclast differentiation. In a previous report (5), we found that RANKL expression in primary human bone marrow adipocytes which was significantly increased by dexamethasone, a critical regulator of osteoclast differentiation, osteoporosis, and adipocyte function. In the present study, we examined 1) the expression of osteoclast differentiation factors, 2) the ability of bone marrow adipocytes to promote osteoclast differentiation and activation by co-culture of bone marrow adipocytes with osteoclast precursors, and 3) the effect of cell-to-cell contact on osteoclast differentiation in co-culture.

MATERIAL AND METHODS

Subjects and isolation of bone marrow adipocytes. During prosthetic replacement surgery of the hip joint, 20 mL of bone marrow fluid was obtained from 33 patients with femoral neck fractures or osteoarthritis. The subjects consisted of 5 males and 28 females, with a mean age of 68.5 (range 52–81, SD ± 9.8) years. We excluded patients with diabetes mellitus, rheumatoid arthritis, or metabolic bone disorders, and those with a history of glucocorticoid therapy. Before surgery, informed consent was obtained. The study protocol was approved by the Institutional Ethics Review Board.

Bone marrow adipocytes were isolated from bone marrow fluid (5). Briefly, bone marrow fluid was mixed with 20 mL of Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY), and treated with 0.1% collagenase A (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C. Digested cells were then centrifuged at 200 × *g* for 5 min, and the adipocyte layer was carefully aspirated from the upper lipid phase. To purify isolated adipocytes, the cells were filtered and washed three times with fresh medium. The adipocytes were counted and 1×10^7 cells were then suspended in 5 mL serum-free DMEM in 15-mL Falcon tubes, and subjected to suspension culture.

RANKL, OPG, and M-CSF m-RNA levels quantified by real-time RT-PCR. Bone marrow adipocytes were cultured in serum-free DMEM with dexamethasone for 12 or 24 h. Isolation of total RNA from bone marrow adipocytes from floating cultures was performed using an RNeasy kit (Qiagen, Hilden, Germany), as per the manufacturer's instructions. RANKL, OPG, macrophage-colony stimulating factor (M-CSF), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expressions were analyzed with 100 ng of total RNA by real-time RT-PCR using a TaqMan One Step PCR Master Mix Reagents kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 7000 Sequence Detector System (Applied Biosystems). PCR amplification was performed with 40 cycles at 95°C for 15 s and 60°C for 60 s. The following PCR primers were used for TaqMan Gene Expression assays at 20 concentration: RANKL: Hs00243519_ml, OPG: Hs00171068_ml, M-CSF: Hs00174164_ml, and GAPDH: Hs99999905_ml (Applied Biosystems). The relative expression between samples was calculated using the comparative cycle threshold (CT) method (ΔCT). Briefly, the formula $X_N = 2^{-\Delta CT}$ was used, where X_N is the relative amount of the target gene in question and ΔCT is the difference between the CT of the gene in question and the CT for GAPDH.

Co-culture of bone marrow adipocytes and osteoclast precursors. We examined the effects of bone marrow adipocytes on osteoclast differentiation in a co-culture system of bone marrow adipocytes and human osteoclast precursors. Human osteoclast precursors (Lonza Walkersville Inc., Walkersville, MD) were cultured in a 9-cm^2 SlideFlask (A/S Nunc, Roskilde, Denmark) with osteoclast precursor growth medium supplemented by 10% FBS, 2 mM L-glutamine, penicillin/streptomycin, and 33 ng/mL M-CSF at a density of $3 \times 10^5/cm^2$ at 37°C in 5% CO_2 for 2 days. After the adhesion of cells to the flask base was confirmed, the flask was completely filled with medium. According to Sugihara's method (18), bone marrow adipocytes ($3 \times 10^5/cm^2$) were seeded onto osteoclast precursors by inverting the flask up-side down for cell-to-cell contact and co-cultured at 37°C in 5% CO_2 for 7 days (with 10^{-7} M dexamethasone or control).

We also performed co-culture under non-contact conditions. Osteoclast precursors were seeded ($3 \times 10^5/cm^2$) in Lab-Tek Chamber Slide 16-well Glass Slides (Thermo Fisher Scientific, Rochester, NY). After 24 h, bone marrow adipocytes were plated on

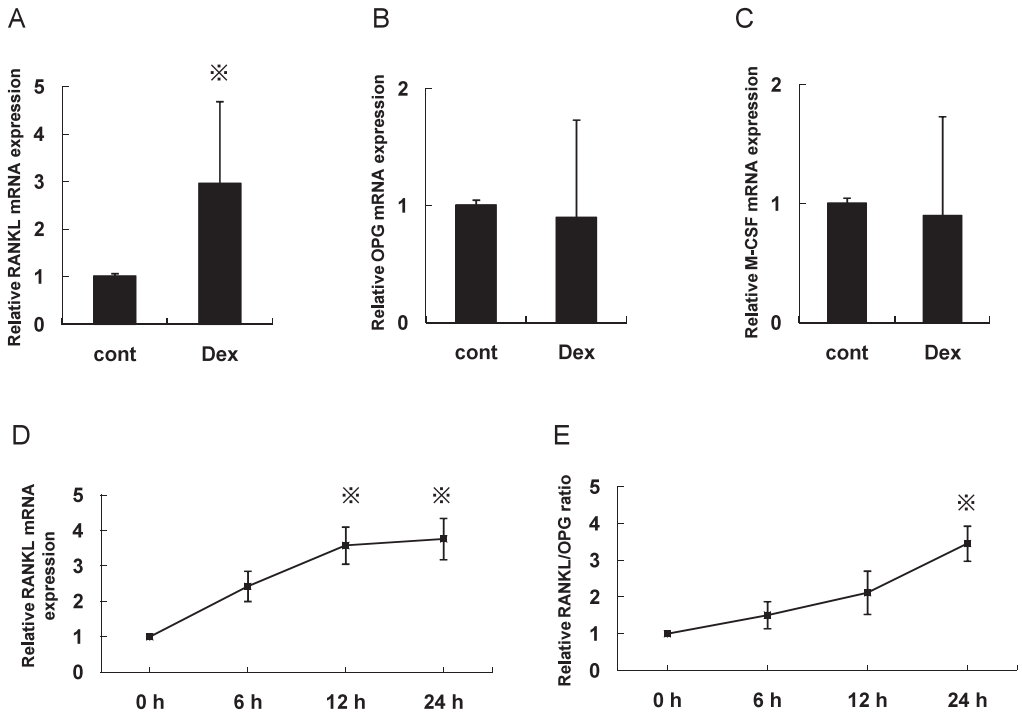

Fig. 1 Effect of dexamethasone on RANKL, OPG, and M-CSF mRNA expression after 12 h of suspension culture of bone marrow adipocytes. (**A**–**C**) Human bone marrow adipocytes were cultured in serum-free DMEM with 10^{-7} M dexamethasone for 12 h, and RANKL, OPG, and M-CSF mRNA levels were quantified by real-time RT-PCR. Dexamethasone significantly induced RANKL mRNA ($P < 0.05$), but not OPG or M-CSF mRNA. (**D**–**E**) Time-course study of RANKL and RANKL/OPG mRNA expression up to 24 h. RANKL mRNA levels increased significantly in bone marrow adipocytes treated with dexamethasone after 24 h (3.8-fold) (D), and the RANKL/OPG ratio was also elevated in the time-course study with dexamethasone treatment at 24 h (3.5-fold) (E). Data represent the relative expression against control levels and are expressed as the mean ± SD of measurements. Asterisks indicate a significant increase ($P < 0.05$) compared to control.

each insert with 0.1-μm pore size membranes (Nunc Tissue Culture Inserts 8-Well Strip (A/S Nunc)) which were located at a height of 1 mm from the bottom of the wells. Plates were cultured at 37°C in 5% CO_2 for 7 days (with 10^{-7} M dexamethasone or control). As a positive control, osteoclast precursor differentiation was performed with osteoclast differentiation medium with 66 ng/mL soluble RANKL for 7 days.

TRAP and Von Kossa contrast staining. To examine osteoclast differentiation in the co-culture, we used a TRAP staining reagent kit (Takara-Bio, Tokyo, Japan). After having fixed the osteoclast precursors, we added 1.25 mL of reaction buffer, including a tartaric acid Na buffer with acid phosphatase, to each slide flask and incubated them at 37°C for 45 min. After having removed the reaction reagents, discs were washed with sterile distilled water three times. The coverslips were examined by light microscopy.

To examine whether the differentiated precursors had the ability of bone resorption, co-culture was performed in culture discs coated by calcium phosphate. Osteoclast precursors ($3 \times 10^5/cm^2$) were cultured with osteoclast precursor growth medium in BD BioCoat Osteologic discs (Becton Dickinson and Co., Franklin Lakes, NJ). Twenty-four hours later, bone marrow adipocytes were seeded at a density of $3 \times 10^5/cm^2$ in the culture discs with 10^{-7} M dexamethasone. Then the discs were completely filled with medium and inverted upside down for cell-to-cell contact. The discs were cultured at 37°C in 5% CO_2 for 7 days. Similarly, co-culture under non-contact conditions was performed with BD BioCoat Osteologic discs with use of the culture insert.

Co-culture discs were stained at day 7 following the removal of cells with trypsin-EDTA (Gibco BRL, Grand Island, NY). Von Kossa contrast staining was performed to evaluate resorption of calcium phosphate films coated on the culture discs. After staining with 5% silver nitrate for 30 min, discs were developed with 5% sodium carbonate in 25% formalin. Two minutes after fixing with 5% sodium

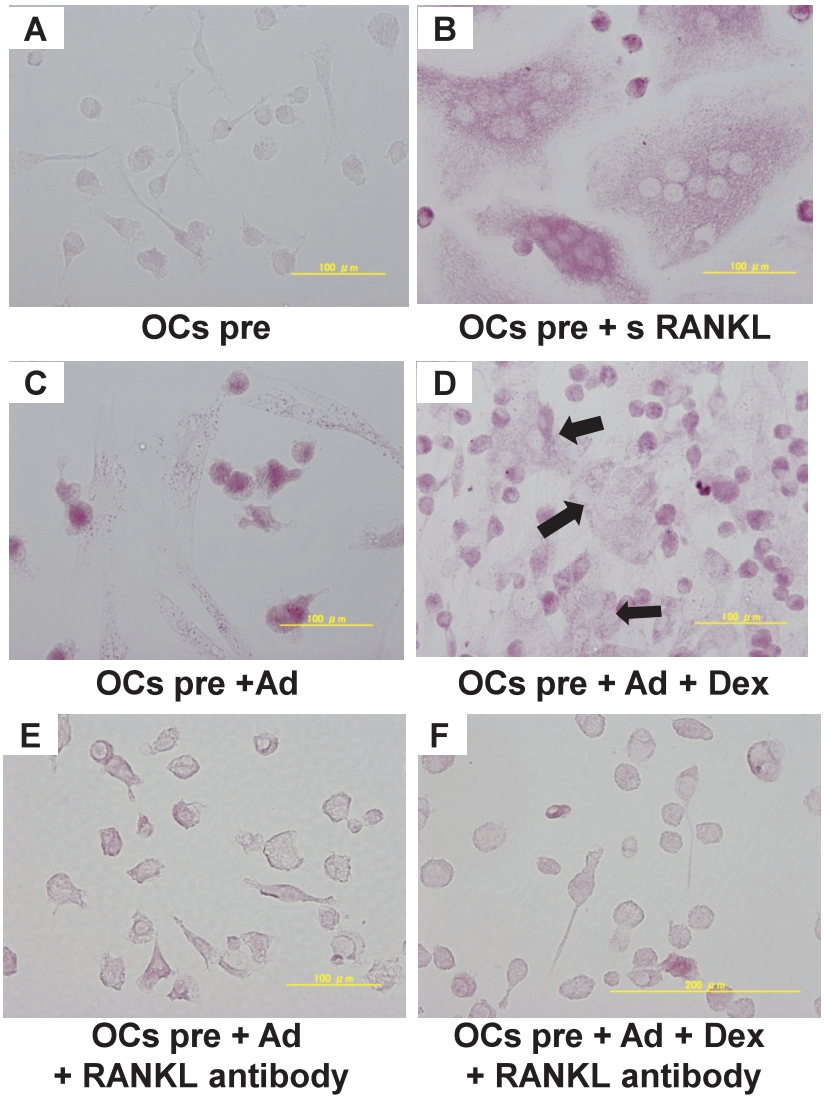

Fig. 2 TRAP staining for osteoclast precursors (OCs pre) after 7 days of co-culture with bone marrow adipocytes (Ad) under cell-contact conditions. (**A**) Negative control: osteoclast precursors without adipocytes or soluble RANKL. (**B**) Positive control: osteoclast precursors with soluble RANKL (66 ng/mL). (**C**) Osteoclast precursors co-cultured with adipocytes. In co-culture without dexamethasone, only 4.2% of TRAP-positive cells were multinuclear. (**D**) Osteoclast precursors co-cultured with adipocytes in the presence of 10^{-7} M dexamethasone (Dex). There was a significant increase in the number of TRAP-positive cells by treatment with dexamethasone (3.4-fold), and 9.3% of TRAP-positive cells became multinuclear (arrows). The multinuclear cell size in co-culture was significantly smaller than that of the positive control. (**E**) Osteoclast precursors co-cultured with adipocytes in the presence of anti-RANKL antibody (1 μg/mL). (**F**) Osteoclast precursors co-cultured with adipocytes in the presence of anti-RANKL antibody (1 μg/mL) and 10^{-7} M dexamethasone. In co-cultures with anti-RANKL antibody treatment, almost no cells differentiated into TRAP-positive cells.

thiosulphate, discs were examined by light microscopy.

Anti-RANKL antibody treatment. To examine the effect of RANKL on osteoclast differentiation, we performed co-culture with anti-RANKL antibody treatment. One μg/mL of monoclonal anti-human RANKL antibody (R&D Systems, Inc., Minneapolis, MN) was added in each flask before co-culture. The culture conditions were identical to the cell-to-cell contact conditions. TRAP staining and Von Kossa staining were also performed to evaluate the differentiation of osteoclast precursors.

Statistical analysis. Results are expressed as the mean value ± SE. Differences between two or more groups were tested by the Mann-Whitney or Kruskal-Wallis test. Relationships with P values less than 0.05 were considered statistically significant.

RESULTS

Primary human marrow adipocytes expressed RANKL, OPG, and M-CSF genes. To study the effect of dexamethasone on the expression of RANKL and OPG in bone marrow adipocytes, cells were treated with 10^{-7} M dexamethasone. Dexamethasone significantly induced RANKL mRNA, but not OPG or M-CSF mRNA (Fig. 1A–C). In a time-course study, RANKL mRNA levels increased significantly in bone marrow adipocytes treated with dexamethasone after 24 h (3.8-fold) (Fig. 1D), and the RANKL/OPG ratio was also elevated in the time-course study with dexamethasone treatment at 24 h (3.5-fold) (Fig. 1E).

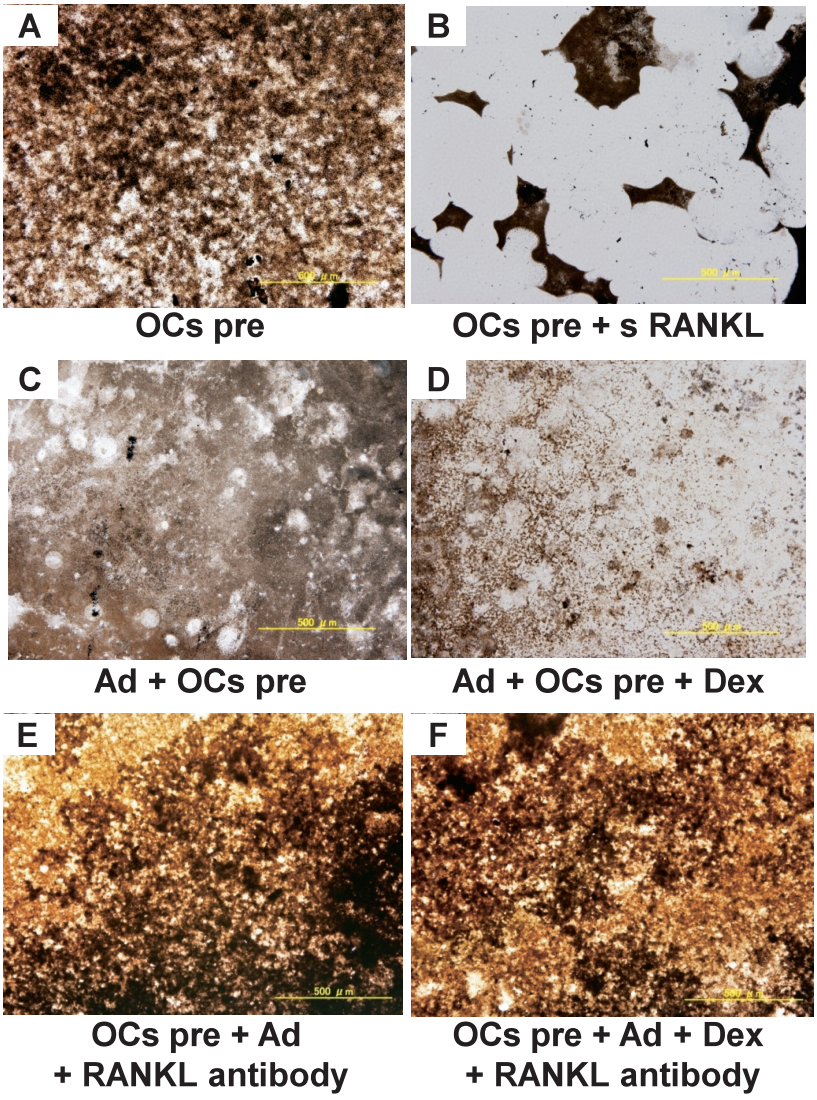

Fig. 3 Von Kossa staining of culture discs coated with calcium phosphate film. Osteoclast precursors (OCs pre) were co-cultured on culture disc coated with calcium phosphate film with bone marrow adipocytes (Ad), using the ceiling culture method under cell-contact conditions. (**A**) Negative control: osteoclast precursors without adipocytes or soluble RANKL. (**B**) Positive control: osteoclast precursors with soluble RANKL (66 ng/mL). (**C**) Osteoclast precursors co-cultured with adipocytes. Resorption cavity formations were found clearly as compared with the negative control. (**D**) Osteoclast precursors co-cultured with adipocytes in the presence of 10^{-7} M dexamethasone. More resorption cavity formations were found compared to the discs without dexamethasone. However, the resorption cavity formation in the co-culture group was smaller as compared with the positive control. (**E**) Osteoclast precursors co-cultured with adipocytes in the presence of anti-RANKL antibody (1 μg/mL). (**F**) Osteoclast precursors co-cultured with adipocytes in the presence of anti-RANKL antibody (1 μg/mL) and 10^{-7} M dexamethasone. Anti-RANKL antibody blocked the cavity formation.

To confirm the differentiation of osteoclast precursors in co-culture with adipocytes, we performed TRAP staining. There was a significant increase in the number of TRAP-positive cells by treatment with dexamethasone (3.4-fold) (Fig. 2D). In co-culture without dexamethasone, only 4.2% of TRAP-positive cells were multinuclear (Fig. 2C). In the presence of dexamethasone, 9.3% of TRAP-positive cells became multinuclear (Fig. 2D). The multinuclear cell size in co-culture was significantly smaller than that of the positive control (Fig. 2B *vs.* 2D). In co-cultures with anti-RANKL antibody treatment, almost no cells differentiated into TRAP-positive cells (Fig. 2E, F).

Co-cultures of bone marrow adipocytes and osteoclast precursors were performed to evaluate the resorption of calcium phosphate films coated on the culture discs. On discs for which co-culture was performed, resorption cavity formations were found clearly as compared with those of single cultured osteoclast precursors (Fig. 3A *vs.* 3C). Moreover, in discs with added dexamethasone, more resorption cavity formations were found compared to the discs without dexamethasone (Fig. 3C *vs.* 3D). However, the resorption cavity formation in the co-culture group was smaller as compared with the positive control with added soluble RANKL (Fig. 3B *vs.* 3C, D). These findings do not contradict the formation of osteoclasts which we confirmed with TRAP staining (Fig. 2).

In co-cultures with non-contact conditions (Fig. 4), no TRAP-positive cells were seen in any group (Fig. 4B, C). Also, no resorption cavity formation was seen in any group (Fig. 4E, F).

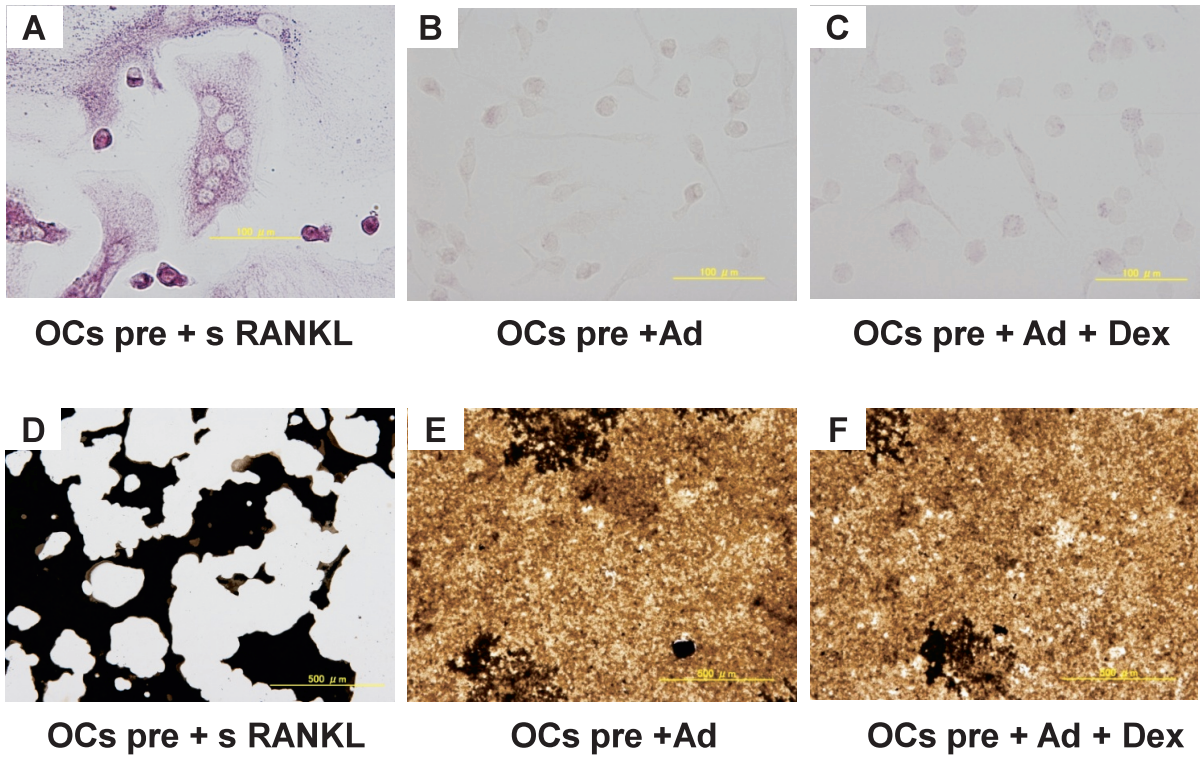

Fig. 4 TRAP (A-C) and Von Kossa staining (D–F) for osteoclast precursors (OCs pre) co-cultured with bone marrow adipocytes (Ad) under non-cell-contact conditions. (**A**, **D**) Positive control: osteoclast precursors were cultured with soluble RANKL (66 ng/mL). (**B**, **E**) Osteoclast precursors were co-cultured with adipocytes. (**C**, **F**) Osteoclast precursors co-cultured with adipocytes in the presence of 10^{-7} M dexamethasone. In co-cultures with non-contact conditions, no TRAP-positive cells were seen in any group. Also, no resorption cavity formation was seen in any group.

DISCUSSION

What is the role of adipocytes in the bone marrow? Hypotheses have been suggested in past reports, such as serving as a simple spacer between hematopoietic cells and bone tissue, an endocrine organ acting on fat metabolism, an energy storehouse, or supporting cells of hematopoiesis and bone metabolism (3, 14). As for adipose tissue, there have been many reports that suggest a relationship between the various cytokines discharged by adipocytes and bone metabolism. Leptin inhibits bone formation through a beta 2 adrenergic receptor (22), or inhibits the formation of osteoclasts and the resorption of bone through the RANKL/RANK/OPG system (4). Moreover, one study has shown that adiponectin increases RANKL and inhibits OPG expression in human osteoblasts through the mitogen-activated protein kinase (MAPK) signaling pathway (10). In overweight postmenopausal women, bone loss is reduced due to estrogen production in adipose tissue (24). Thus, adipocytes have a relation to bone metabolism through secreting adipokines.

There have also been many studies showing active functioning for bone metabolism in bone marrow adipocytes. Weisberg *et al.* suggested that bone marrow adipocytes not only suppress osteoblastogenesis, but might promote bone resorption because bone marrow adipocytes secrete inflammatory cytokines capable of recruiting osteoclasts (27). BMS2 adipocytes, a marrow stromal cell line, promote the differentiation of osteoclast-like cells in the presence of vitamin D (7). Bone marrow stromal cell lines having a high potential for osteoclast-supporting activity express peroxisome proliferator activated receptor γ 1 (PPARγ1) and show high potential for differentiation into adipocytes (20). Additionally, 3T3-L1 adipocytes and rat adipose tissue have been shown to express RANKL, an essential molecule in osteoclast differentiation, and the decoy receptor molecule OPG (2). Bone marrow adipocytes are associated with bone resorption by affecting osteoblast/osteoclast differentiation.

RANKL is an essential molecule in osteoclast differentiation, as is decoy receptor molecule OPG. Marrow stromal cell-derived osteoblasts can support

osteoclast differentiation through RANKL expression (13). Osteoblasts and marrow stromal cells stimulate osteoclast differentiation depending on stimulation by various inflammatory cytokines, PTH, vitamin D, or glucocorticoids (9, 28). Also, osteoblasts and marrow adipocytes have a common progenitor, mesenchymal stem cells (13). Hozumi *et al.* have reported that primary human mature bone marrow adipocytes express not only these cytokines but also key molecules of bone metabolism itself, such as RANKL and OPG, and that RANKL expression is stimulated by glucocorticoids (5). The results mentioned above show that bone marrow adipocytes may be associated with osteoclast differentiation directly, the same as with osteoblasts and marrow stromal cells.

In this study, osteoclast precursors differentiated to mature osteoclasts that were TRAP-positive and had the ability of bone resorption, as shown by co-culturing primary human bone marrow adipocytes and osteoclast precursors in the presence of M-CSF. We suggest that primary human mature bone marrow adipocytes express RANKL, and that it is possible that bone marrow adipocytes are associated with osteoclast differentiation directly. Although the size of osteoclasts in co-culture was smaller than in the positive control, and the number of multinuclear cells was small, the cells were TRAP-positive and had the ability to resorb calcium phosphate films, so we recognized them as differentiated osteoclasts. In the first stage of the osteoclast differentiation process, osteoclast precursors become mononuclear TRAP-positive cells, and then they become multinuclear giant cells as a result of cell fusion (26). In the present study, we found that the RANKL mRNA expression of adipocytes was less intense as compared with a positive control in co-culture, so the osteoclast precursors remained in a differentiation stage preceding the multinucleate giant cells.

In contrast, the osteoclast precursors did not differentiate in the co-culture under non-cell contact conditions using cell culture inserts. These results show that direct contact with osteoclast precursors and bone marrow adipocytes is necessary for osteoclast differentiation, as with osteoblasts; cell-to-cell contact of osteoblasts and osteoclast precursors is important for the formation of osteoclast-like cells (21, 25). RANKL expressed on the surface of bone marrow adipocytes may signal to osteoclast precursors to differentiate into mature osteoclasts. In co-cultures with anti-RANKL antibody treatment, osteoclast precursors did not differentiate into TRAP-positive cells and could not resorb bone. These results indicate that RANKL expressed by bone marrow adipocytes promotes osteoclast differentiation in co-culture under cell-to-cell contact conditions.

Additionally, osteoclast differentiation was promoted in co-culture by stimulation with dexamethasone. This is presumably due to enhancement of the ability of bone resorption by an increase in RANKL expression in bone marrow adipocytes. It is known that glucocorticoids cause osteoporosis, and the mechanism of action is recognized as follows: suppression of Ca^{2+} absorption in the small intestine, suppression of Ca^{2+} resorption in the renal tubule, a decrease of hormone secretion through the pituitary adrenal system, suppression of bone formation by the direct action of osteoblasts, and the promotion of bone resorption by the direct action of osteoclasts (16). In addition to these factors, the promotion of osteoclast differentiation by RANKL expression increases the effect of bone marrow adipocytes, and may be associated with the onset and exacerbation of osteoporosis. Bone marrow adipocytes increase in patients with osteoporosis (3, 11, 12, 19). There is a viewpoint that this increase is a result of a changed differentiation ratio of mesenchymal stem cells (3), but considering the results of this study, it seems that the effects of glucocorticoids on bone marrow adipocytes are positively associated with the onset and exacerbation of osteoporosis as well.

Our study was limited by the fact that the specimens were harvested from patients with osteoarthritis of the hip or femoral neck fracture during prosthetic replacement surgery of the hip joint, so these diseases may have influenced the results. Moreover, the age and sex of the patients, physique, and bone density were not constant, and there may have been individual differences in their response (6, 29). However, these issues do not cause us to doubt that factors related to bone metabolism are secreted by bone marrow adipocytes, and that these may have a significant effect on the body.

In conclusion, we found that the primary human bone marrow adipocytes express RANKL similar to osteoblasts, and that they stimulate osteoclast differentiation and activity in a manner directly related to RANKL expression. These results suggest that bone marrow adipocytes are important in bone metabolism, and that they are related to bone resorption in steroid osteoporosis.

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