Potentiation of osteoclastogenesis by adipogenic conversion of bone marrow-derived mesenchymal stem cells

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ABSTRACT

Bone marrow-derived mesenchymal stem cells (BMSCs) are the indispensable component of the bone marrow, being the common precursors for adipocytes and osteoblasts. We show here that adipogenic differentiation resulted in increase in the production of adipocyte markers, such as adiponectin, fatty-acid binding proteins (FABP4), peroxisome proliferator-activated receptor γ (PPARγ), as well as the receptor activator of nuclear-κB ligand (RANKL). Co-culture of osteoclast precursors (OCPs) with BMSCs-derived adipocytes significantly enhanced osteoclast differentiation with low-dose RANKL, whose levels alone could not promote osteoclastogenesis. These results demonstrate for the first time that adipogenic differentiation of BMSCs plays a pivotal role in maintaining bone homeostasis.

Bone homeostasis is maintained by various types of cells, such as osteoblasts and osteoclasts, which are differentiated from the different stem cells in the bone marrow (2, 4, 33, 34). While osteoclasts are derived from hematopoietic stem cells (HSCs), osteoblasts are differentiated from bone marrow-derived mesenchymal stem cells (BMSCs). Therefore, harmonized coordination of the activities of these two stem cell compartments in the bone marrow is indispensable for bone homeostasis. In contrast, bone remodeling is regulated by the balance between osteogenesis and osteoclastogenesis (12, 14, 30, 32, 36). Osteoclasts are the central player in osteoclastogenesis, which are derived from monocytes in contact with osteoblasts through the interaction of receptor activator of nuclear-κB on osteoclast precursors and the receptor activator of nuclear-κB ligand (RANKL) expressed on osteoblasts (1, 13, 22, 26, 35). As a consequence, osteogenic differentiation from BMSCs is now recognized as an essential part integrated into bone remodelling.

Accumulating evidences have also suggested that adipogenesis could play a significant role in bone metabolism (5, 8, 9, 17, 24, 28, 29). For example, since adipocytes and osteoblasts have the same ancestor, osteoblastogenesis and adipogenesis in the bone marrow are regulated in an opposite way, indicating that adipogenic conversion results in a reduction in osteoblast pool (24). As an endocrine organ, adipose tissue secretes adipokines, such as leptin and adiponectin, which may suppress the functions of osteoblasts (5, 17, 18). More recently, age-related fat accumulation and osteoporosis have been extensively discussed, and it is hypothesized that age-related bone marrow adiposity could accelerate osteoclastogenesis (3, 17, 27, 31, 38). Thus, it is highly likely that adipocytes in the bone marrow may have a positive interaction in osteoclastogenesis. In fact, our recent studies demonstrated that the primary human bone marrow adipocytes stimulate TNF-α or dexamethasone-induced osteoclast differentiation, which

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is mediated by augmented expression of RANKL in adipocytes (10, 11, 15).

These lines of evidence also suggest that adipogenic differentiation of BMSCs in the bone marrow might stimulate osteoclastogenesis. In the present study, we examined whether adipogenic differentiation of BMSCs physiologically promotes osteoclast differentiation. We observed that adipogenic differentiation from BMSCs triggered production of adiponectin, fatty-acid binding proteins (FABP4), peroxisome proliferator-activated receptor γ (PPARγ), as well as RANKL. This study is the first to show that co-culture of osteoclast precursors with BMSCs-derived adipocytes significantly enhanced osteclast differentiation in a RANKL dose-dependent manner. These results ensure that BMSCs play a pivotal role in maintaining bone homeostasis by both osteoblastogenesis-mediating bone formation and adipogenesis-stimulating bone resorption.

MATERIALS AND METHODS

Cultivation of BMSCs. Human bone marrow-derived mesenchymal stem cells (BMSCs) were obtained from Lonza Japan (Lonza Japan Co. Ltd., Tokyo), and cells were cultured in the mixture of the special Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical Co. Ltd., Tokyo), optimized for primary human cell culture, supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific, Victoria, Australia) and ES cell medium (ReproCELL Inc., Tokyo) (4 : 1). Cells were subcultured every 3 to 4 days to keep them in an exponentially growing state.

Adipogenic differentiation of BMSCs. BMSCs (2×10^4 /cm^2) were maintained in MEM supplemented with 10% FBS for five days to obtain confluence. Then, culture medium was changed to Adipocyte Differentiation Medium (DM-2) (Zen-Bio inc., Durham), and cells were incubated for 14 days in a 5% CO_2 incubator at 37°C. DM-2 medium was exchanged every three days. The formation of lipid droplets was checked under the phase-contrast microscope.

To confirm adipogenesis, a part of BMSCs was cultured on glass cover slips, and incubated in DM-2 medium for 14 days. Cells were then fixed in a 4% formalin for 20 min at room temperature. Then, cells were incubated with 5 μM of BODIPY 493/503 (Life Technologies Co., Grand Island) for 30 min. After washing cells with PBS, cells were stored in PBS containing 10% glycerol, and the cover slips were sealed onto the slide glass. The BODYPI staining was examined under a fluorescence microscope, and digital images were obtained using FW4000 software (Leica Microsystems, Tokyo).

Differentiation of osteoclast precursors. Human osteoclast precursors (OCPs) (Lonza Japan, Co. Ltd.) were cultured in an osteoclast precursor growth medium (Lonza Japan, Co. Ltd.). For osteoclast differentiation, OCPs (2×10^3 cells/well) were seeded onto the Osteo assay 24-well plates (CORNING Inc., Lowell) in an osteoclast defferentiation medium, which is an osteoclast precursor growth medium containing 33 ng/mL M-CSF and 66 ng/mL RANKL. The cells were incubated for 7 days in a CO_2 incubator at 37°C. The formation of multinucleated osteoclasts was checked under the phase-contrast microscope.

Co-culture of OCPs and adipocytes differentiated from BM-MSCs. OCPs (2×10^3 cells/well) seeded on the Osteo assay well plates were incubated for 24 h, and then, they were co-cultured with adipocytes (3×10^5 cells/well) differentiated from BMSCs for 14 days. The cells were incubated for further 7 days in an osteoclast precursor growth medium containing 33 ng/mL M-CSF and RANKL at the concentration between 0 and 24.8 ng/mL.

von Kossa staining. Cells in 24-well plates were fixed with 10% formalin for 10 min and they were placed in 3% silver nitrate solution containing 5% NaHCO$_3$. After exposing well plates to ultraviolet lights (UVC) for 1 h, images of each well were obtained by digital camera. Bone resorption was quantified by measuring the white areas using image-processing software.

Western blotting analysis. BMSCs were incubated in a DM-2 medium for 14 days. Differentiated cells were collected and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate and 0.1% sodium dodecylsulfate) containing protease inhibitor cocktail (Roche Diagnostics Japan, Co. Ltd., Tokyo). Western blotting was performed as previously described. Briefly, the cell lysate was cleared by centrifugation at 15,000 rpm for 10 min at 4°C. Then, the supernatant was collected and used as total cellular protein. Total protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). Proteins (8 or 16 μg) were electrophoresed through an SDS-polyacrylamide gel and were electrophoretically

transferred to a polyvinyl difluoride membrane in a transfer buffer (100 mM Tris, 192 mM glycine). After overnight incubation with blocking solution (10% skim milk), the membrane was incubated with the primary antibodies, biotinylated anti-mouse or rabbit IgG antibodies, and streptavidin-alkaline phosphatase. To visualize the bands, the membrane was incubated in a detection solution containing nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as a substrate. Primary antibodies used in this study are anti-human adiponectin (clone 19F1; Abcam Co. Ltd., Tokyo), anti-human FABP4 (Abcam Co. Ltd.), anti-human PPARγ (clone 81B8; Cell Signaling technology Japan), anti-human RANKL (Epitomics Inc., Burlingame), anti-human M-CSF (Epitomics Inc.), and anti-human OPG (Epitomics Inc.). The experiments were repeated at least three times.

Statistical analysis. Data were shown as mean ± SD and analyzed for statistical significance using Wilcoxon rank test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Exponentially growing BMSCs were maintained at high density for five days to obtain a confluent culture, and then, they were treated with adipocyte differentiation DM-2 medium for 14 days. By phase contrast images, BMSCs showed spindle-shaped morphology representative of mesenchymal cells (Fig. 1A). After 14 days-treatment, a large number of lipid droplets (Fig. 1B), which were positive for BODIPY493/503 staining, occupied the cytoplasm in approximately 70% of the cells (Fig. 1G). Time-course examination revealed that BODIPY493/503-positive droplets became detectable by day 3 (Fig. 1E). While approximately 30% of cells were positive (Fig. 1H), only a few droplets per cells were induced at this time. At day 7, more than 50% of cells were positive (Fig. 1H), and the number of droplets per cell increased gradually (Fig. 1F). Significant amount of droplets were formed in approximately 70% of cells by 14 days-treatment (Fig. 1G, H), and no significant change was observed for further treatment. Therefore, we decided to use 14 days-treatment with DM-2 medium for adipogenic conversion of BMSCs.

Western blot analysis demonstrated that augmented expression of PPARγ became detectable by day 3, which was followed by the expression of FABP4 and adiponectin. Since the expressions of PPARγ, FABP4 and adiponectin were not detectable by day 0, quantitative analysis of relative expression could not be
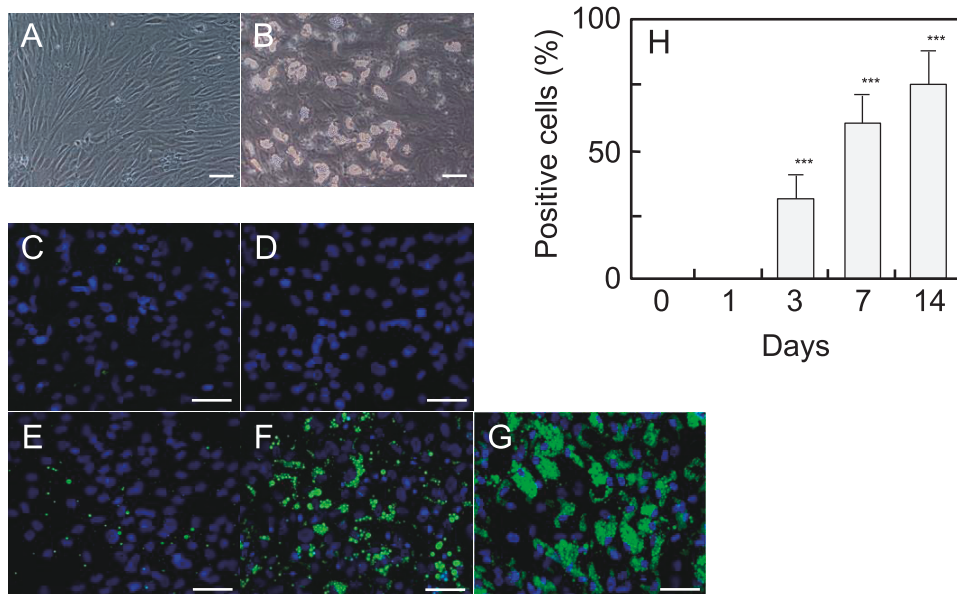

Fig. 1 Adipogenic conversion of BMSCs. BMSCs (3×10^5 cells/cm^2) were incubated for 5 days to get confluent culture. Then, culture medium was replaced with adipogenic differentiation medium (DM-2), and incubated for further 14 days. Phase-contrast images of BMSCs (**A**) and adipocytes at day 14 (**B**). Cells fixed in 4% formalin were incubated for 20 min in PBS containing 5 μg/mL BODIPY493/503 (green), and counterstained with 0.1 mg/mL of DAPI (blue) (**C**–**G**). **C**, Day 0. **D**, Day 1, **E**, Day 3, **F**, Day 7, **G**, Day14. Percentages of cells with BODIPY493/503-positive lipid droplets are determined (**H**). ***, $P < 0.01$ compared with Day 0. White bars indicate 50 μm.
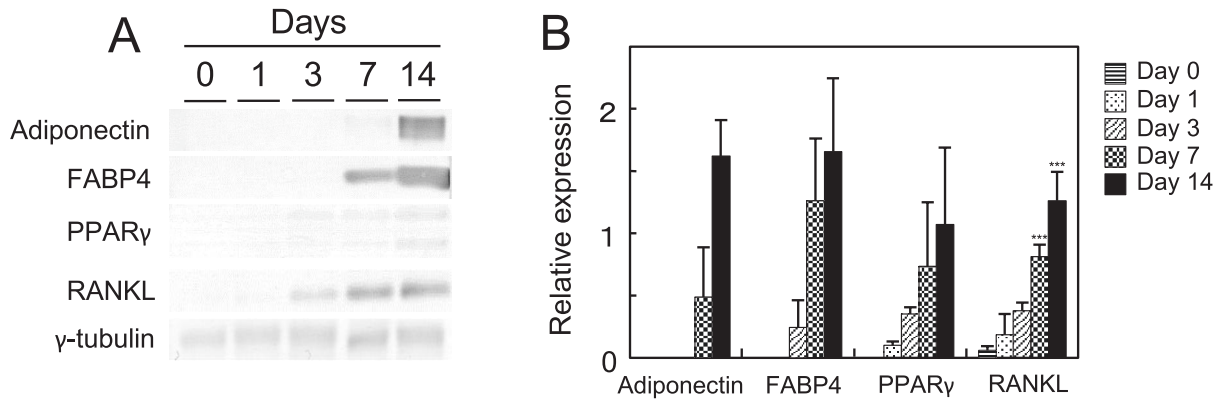
Fig. 2 (**A**) Western blot analysis of protein expressions. Confluent BMSCs were treated with the DM-2 medium for 14 days, and the total cell lysates were obtained at days as indicated. Blots were probed with antibodies as indicated. (**B**) Quantitative analysis of relative expression. The band density was quantified using Image J software, and relative expression to γ-tubulin expression was caluculated. ***, $P < 0.01$ compared with Day 0.

possible. In contrast, enormous levels of PPARγ, FABP4 and adiponectin proteins were observed at day 14 (Fig. 2A), confirming that adipogenic conversion of BMSCs was sufficiently induced by 14 days-incubation in adipogenic differentiation medium. While RANKL expression was marginally detectable in BMSCs, augmented protein levels of RANKL became obvious by day 3 (Fig. 2A). We failed to detect expressions of M-CSF and OPG (data not shown). Quantitative analysis of relative expression demonstrated that significant up-regulation of RANKL expression was confirmed in BMSCs-derived adipocytes by day 7 (Fig. 2B). After day 7, as BMSC converted adipocytes, RANKL expression increased significantly.

Involvement of adipocyte conversion of BMSCs in osteoclastogenesis was examined in osteoclast precursors (OCPs) co-cultured with adipocytes differentiated from BMSCs. Since osteoclast differentiation medium originally contains 66 ng/mL of RANKL, we determined the RANKL concentration, by which osteoclast differentiation was not taken place. As shown in Fig. 3, osteoclast differentiation, which was quantified by bone resorption evaluated by von Kossa staining, was not observed without RANKL (Fig. 3G), whereas 66 ng/mL of RANKL showed approximately 30% bone resorption without adipocytes (Figs. 3H and 4). Little or no bone resorption was observed with 24.8 ng/mL of RANKL even in the presence of BMSCs (Fig. 3A). In contrast, we found that OCPs co-cultured with adipocytes efficiently promoted osteoclast differentiation with 8.3 ng/mL of RANKL (Fig. 3D). Although the promoting effects were not evident with RANKL at doses of 4.1 ng/mL or lower, adipocytes supported

RANKL dose-dependent increase in bone resorption (Fig. 4).

DISCUSSION

BMSCs have been known for many years to have the potential to differentiate into multiple cell types, including osteoblasts and adipocytes (2, 4, 33, 34). While the physiological role of osteoblasts in bone metabolism is well described, a role of adipocytes in the bone marrow is still uncertain. Recently, several reports have suggested a possible connection between age-related fat accumulation and osteoporosis, indicating that accelerated adipogenesis might exaggerate physiological osteoclastogenesis (3, 17, 27, 31, 38). Considering that BMSCs are the precursors of osteoblasts, adipogenesis in the bone marrow limits the pool of osteoblasts, however, recent studies have indicated that it also potentiates bone metabolism through secretion of the factors such as insulin, insulin-like growth factor, and leptin (5, 17, 18, 24). In fact, our previous observations demonstrated that bone marrow-derived adipocytes expressed the RANKL gene transcripts, whose level was up-regulated by TNF-α and dexamethasone (10, 11, 15). Therefore, in the present study, we aimed to examine the role of adipogenic conversion of BMSCs in osteoclastogenesis *in vitro*, focusing on the production of RANKL.

We found that adipogenic differentiation of BMSCs significantly stimulates the synthesis of RANKL proteins, which is in agreement with recent observation (16). While BMSCs by themselves showed very low levels of RANKL production, noticeable up-regulation was observed at least three days incubation
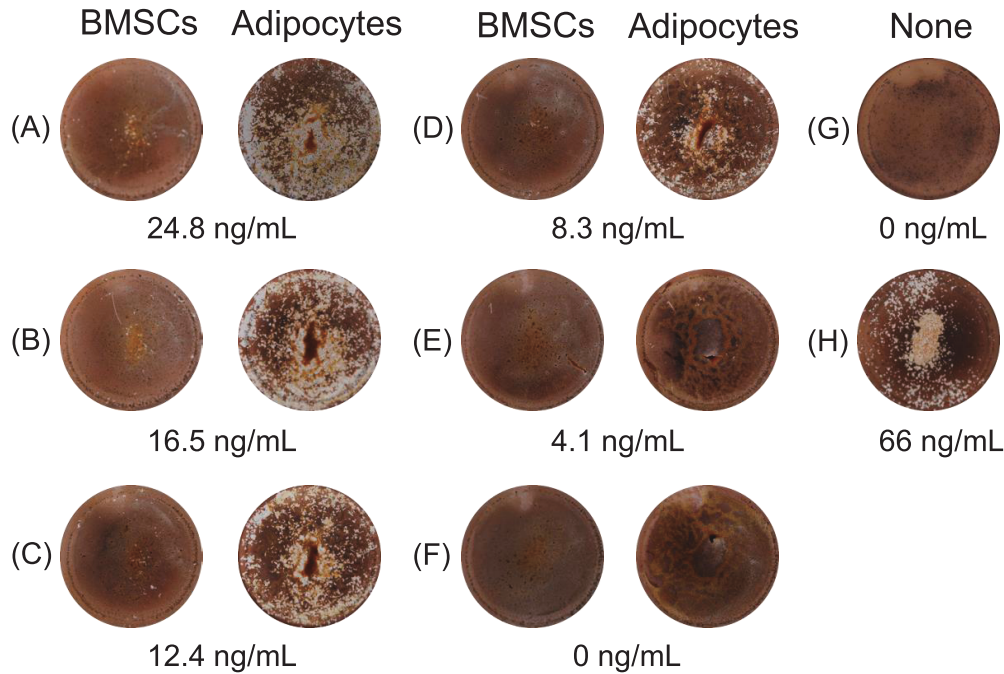
Fig. 3 von Kossa staining of OCPs. OCPs seeded on the Osteo assay well plates were co-cultured in the osteoclast differentiation medium for 7 days with BMSCs (**A**–**F**, left columns) or with adipocytes differentiated from BMSCs (**A**–**F**, right columns). The osteoclast differentiation medium contains M-CSF (33 ng/mL) and RANKL at a concentration of 24.8 ng/mL (**A**), 16.5 ng/mL (**B**), 12.4 ng/mL (**C**), 8.3 ng/mL (**D**), 4.1 ng/mL (**E**), and 0 ng/mL (**F**). OCPs were cultured in the osteoclast differentiation medium for 7 days in the presence of 33 ng/mL of M-CSF and 0 ng/mL (**G**) or 66 ng/mL (**H**) of RANKL. The wells were fixed with 10% formalin for 10 min, placed in 3% silver nitrate solution containing 5% sodium bicarbonate, and exposed to UVC (0.25 J/m^2) for 1 h. The bright area in the wells were judged as resorption pits.

of BMSCs in an adipocyte differentiation medium. Importantly, as shown in Fig. 1, induction of PPARγ and FABP4 was barely detectable by day 3, indicating that RANKL up-regulation was executed before the adipogenic conversion became obvious. Our previous studies demonstrated that dexamethasone could stimulate the RANKL gene expression in bone marrow adipocytes, and the adipogenic differentiation medium contains dexamethasone (11, 15). However, another study reported that RANKL expression was not affected by dexamethasone in bone marrow stromal cells (20). Thus, the mechanism, by which adipogenic conversion of BMSCs stimulates RANKL expression, remains to be determined, but it can be concluded that the commitment of BMSCs to adipocytes is mutually associated with the up-regulation of RANKL expression.

Since RANKL is a critical factor for osteoclast differentiation (21, 22, 26), it is possible that adipogenic conversion of BMSCs promotes the differentiation of OCPs. In fact, we proved for the first time that BMSCs-derived adipogenesis efficiently promoted osteoclast differentiation *in vitro*. It should be noted that the effect was dependent upon the RANKL
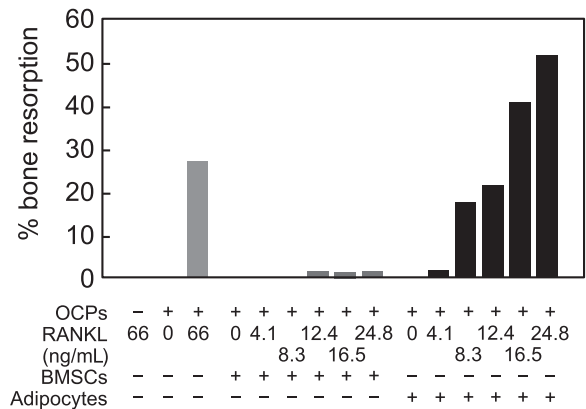

Fig. 4 Quantitative evaluation of von Kossa staining. The well images were obtained by a digital camera, and the percentage of bone resorption was calculated using Image J software. White areas were calculated as bone resorption pits. The threshold was chosen so that the well with a complete dark area was determined to be 0% resorption.

dose in the culture medium. Our results demonstrated that co-culture of adipocytes with OCPs in a medium without RANKL failed to stimulate osteoclast differentiation. Bone resorption was obviously de-

tected with the RANKL dose of 8.3 ng/mL or higher in the presence of adipocytes. Although we failed to detect M-CSF and OPG expressions, a previous study reported that adipogenesis of BMSCs did not change the level of OPG (16), suggesting that the RANKL/OPG ration is also up-regulated.

Our present study clearly demonstrates that adipogenic conversion of BMSCs is able to accelerate osteoclastogenesis, however, previous studies indicated that adipocytes might not be the major source of RANKL. It is well established that osteoblasts differentiated from BMSCs also express RANKL (13, 22, 26). More recently, RANKL provided by osteocytes has been shown to play a critical role in osteoclastogenesis (19, 25, 37). Thus, under a physiological condition, the effect of adipogenesis of BMSCs on osteoclastogenesis may be rather limited. However, our results draw more attention in relation to age-related marrow adiposity, which is now recognized as a common features associated with age-related bone loss (3, 27, 31, 38). Studies have shown that bone marrow stromal cells are more committed to adipogenesis with age (23). Marrow stromal cells obtained from postmenopausal osteoporotic patients displayed pronounced tendency to adipogenic differentiation (6, 7). These observations raise the possibility that unbalanced adipogenesis deliver unfavourable levels of RANKL. Because predominant adipogenic conversion of BMSCs also reduces osteoblast pool in the bone marrow, these could be clues to explain mutual relationship between age-related adiposity and osteoporosis.

In summary, our results demonstrated that BMSCs are indispensable components that regulate bone homeostasis. In addition, bone marrow derived-adipocytes are no more a mere filler for the bone marrow space, rather they promote osteoclastogenesis via RANKL up-regulation. This indicates that adipogenesis in the bone marrow plays a critical role in the regulation of bone resorption.

Acknowledgements

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