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Effect of interferon-γ on the fusion of mononuclear osteoclasts into bone-resorbing osteoclasts

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Osteoclasts are multinucleated cells that are formed by the fusion of pre-fusion osteoclasts (pOCs). The fusion of pOCs is known to be important for osteoclastic bone resorption. Here, we examined the effect of IFN-γ on the fusion of pOCs. IFN-γ greatly increased the fusion of pOCs in a dose-dependent manner. Furthermore, IFN-γ induced pOC fusion even in hydroxyapatite-coated plates used as a substitute for bone. The resorption area of pOCs stimulated with IFN-γ was significantly higher than that of the control cells. IFN-γ induced the expression of dendritic cell-specific transmembrane protein (DC-STAMP), which is responsible for the fusion of pOCs. IFN-γ enhanced DC-STAMP expression in a dose-dependent manner. The mRNA expression of c-Fos and nuclear factor of activated T cells (NFAT) c1 was enhanced in the pOCs treated with IFN-γ. Taken together, these results provide a new insight into the novel role of IFN-γ on the fusion of pOCs. [BMB reports 2012; 45(5): 281-286]

INTRODUCTION

The bone is a dynamic tissue that is maintained by a delicate balance between the potentials of two major cells, osteoclasts and osteoblasts, both of which are exquisitely regulated by the concerted actions of various factors, including estrogen and cytokines (1, 2). Osteoclasts, tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells, originating from hematopoietic cells, are responsible for bone resorption. In contrast, osteoblasts originate from mesenchymal cells and are responsible for bone formation (3). Although osteoclasts and osteoblasts have opposite effects on bone homeostasis, osteoblasts can regulate osteoclast differentiation and function through the induction of a receptor activator of nuclear factor κB ligand (RANKL; also known as ODF, OPGL, and TRANCE) (4-6). Osteoblasts and osteoclasts are known to function antagonistically, but the mechanism underlying this function remains unclear. The balance between osteoclasts and osteoblasts is easily disturbed by systemic alterations, such as chronic inflammation and estrogen deficiency, which increase osteoporosis and, in turn, the risk of fractures (7).

RANKL, which belongs to the tumor necrosis factor (TNF) superfamily, is expressed on the surface of osteoblasts/stromal cells or secreted by activated T cells (5, 6, 8). RANKL directly binds RANK on osteoclast precursors and stimulates multiple intracellular signaling proteins, including NF-κB, c-Fos, and a nuclear factor of activated T cells (NFAT) c1, thereby leading to osteoclast differentiation (9, 10). In addition, RANKL triggers calcium signaling by phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail of both DNAX-activating protein (DAP) 12 and γ chain of Fc receptor (FcRγ) signaling pathways (11, 12). ITAM-dependent calcium signaling is important for the induction of NFATc1 and is indispensable for osteoclast differentiation (13). In particular, NFATc1 is considered to be a master transcription factor that regulates osteoclast differentiation by inducing the expression of osteoclast-specific genes, such as TRAP, osteoclast-associated receptor (OSCAR), and d2 isoform of vacuolar (H^+) ATPase (v-ATPase) V(0) domain (Atp6v0d2) (14, 15).

The formation of multinuclear osteoclasts involves several processes, such as commitment to TRAP-positive cells, fusion to enable multinucleation, and activation to enable bone resorption (1, 3). In particular, multinucleation of pre-fusion osteoclasts (pOCs) is an essential process in efficient bone resorption because pOCs have limited ability of bone resorption (14). Previous reports have shown that CD44, E-cadherin, meltrin-α, Atp6v0d2, and dendritic cell-specific transmembrane protein (DC-STAMP) are required for cell-cell fusion of pOCs (16-20). Recent studies have also shown that a deficiency of DC-STAMP or Atp6v0d2 causes a defect in pOC fusion, there-

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by causing osteopetrosis in mice (19, 20). Thus, cell-cell fusion is an important process in bone resorption.

Osteoclast differentiation is exquisitely regulated by various cytokines, including RANKL, interleukins, interferons (IFNs), TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (6). We have reported recently that although GM-CSF greatly inhibits osteoclast differentiation, it can induce the fusion of pOCs into bone-resorbing osteoclasts (21). These findings indicated that although negative regulators of osteoclast differentiation can inhibit commitment into TRAP-positive cells, they might positively regulate other processes, such as cell-cell fusion and bone resorption.

IFN-β strongly inhibits osteoclast differentiation by suppressing c-Fos expression (22). Furthermore, IFN-γ negatively regulates osteoclast differentiation through the degradation of TNF receptor-associated factor 6 (TRAF6) (23). However, IFN-γ positively regulates osteoclast formation *in vivo* via T cell activation (24). Importantly, this suggests the possibility of IFN-γ having different effects under certain experimental conditions. The effect of IFN-γ on the fusion of pOCs has not been reported yet. This study aimed to examine the effect of IFN-γ on the fusion of pOCs.

RESULTS

IFN-γ enhances the fusion of pOCs into multinucleated osteoclasts

IFN-γ strongly inhibits osteoclast differentiation (23). However, it remains unclear whether IFN-γ regulates the fusion of pOCs and bone resorption. Therefore, to examine the effect of IFN-γ on the fusion of pOCs into multinucleated osteoclasts, we cultured BMMs for 3 days to promote their differentiation into pOCs. More than 90% of the BMMs differentiated into pOCs when they were treated with M-CSF and RANKL for 3 days in an established culture system. The pOCs were stimulated for 12 h with increasing concentrations of IFN-γ. IFN-γ significantly induced the fusion of pOCs into multinucleated osteoclasts (Fig. 1A). Next, we examined whether IFN-γ used in this experiment exerts its anti-osteoclast differentiation activity. IFN-γ strongly inhibits the differentiation of BMMs into osteoclasts (Fig. 1B). These results suggest that although IFN-γ is known to inhibit osteoclast differentiation, IFN-γ can induce the differentiation of pOCs into multinucleated osteoclasts.

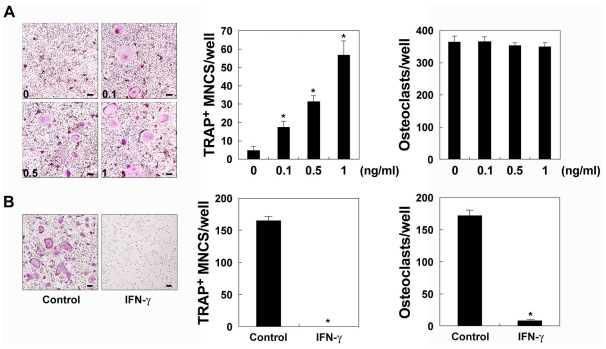

Fig. 1. Effect of IFN-γ on the fusion of pOCs. BMMs were cultured for 3 days with M-CSF (20 ng/ml) and RANKL (50 ng/ml) to induce the formation of pOCs. (A) The pOCs were stimulated for 12 h with increasing concentrations of IFN-γ. The pOCs treated with IFN-γ were stained for TRAP. Scale bar, 100 μm. After staining, the TRAP-positive cells containing 5 or more nuclei were considered as TRAP-positive multinucleated cells ($TRAP^+$ MNCs, middle). The TRAP-positive cells containing 4 or less nuclei were considered as osteoclasts (right). Asterisks indicate $P < 0.01$ versus control (without IFN-γ). (B) BMMs were cultured for 4 days with M-CSF (20 ng/ml) and RANKL (50 ng/ml) in the presence of IFN-γ (1 ng/ml). After culture, the cells were stained for TRAP. TRAP-positive cells were counted as described above. Asterisk indicates $P < 0.01$ versus control (without IFN-γ). Scale bar, 100 μm

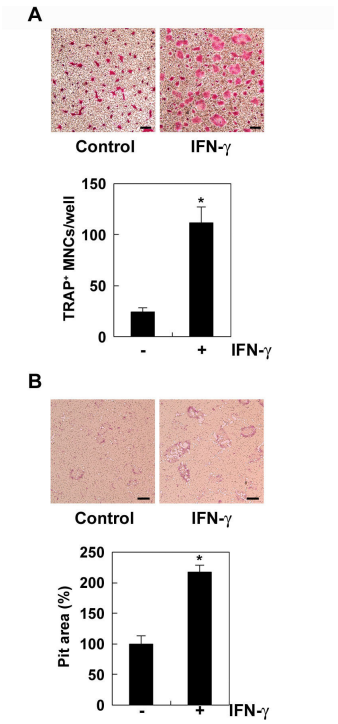

Fig. 2. Effect of IFN-γ on bone resorption of pOCs. (A) BMMs were plated on hydroxyapatite-coated 48-well plates and cultured for 3 days with M-CSF (20 ng/ml) and RANKL (50 ng/ml) to induce the formation of pOCs. The pOCs were stimulated for 12 h with IFN-γ (1 ng/ml) in the presence of M-CSF (20 ng/ml). After 12 h, the cells were stained for TRAP. The stained cells were photographed under a light microscope. Scale bar, 100 μm. TRAP-positive cells containing 5 or more nuclei were considered as TRAP-positive multinucleated cells ($TRAP^+$ MNCs). (B) To examine the effect of IFN-γ on bone resorption, the cells were removed and the plates were photographed under a light microscope. Scale bar, 100 μm. The resorption area was quantified by using the Image Pro-plus program, version 4.0. Statistically significant differences ($P < 0.01$) are marked by an asterisk.

IFN-γ induces the differentiation of pOCs into bone-resorbing osteoclasts

The fusion of pOCs is important for bone resorption (19, 20). Therefore, it is important to determine whether multinucleated osteoclasts induced by IFN-γ could resorb bone. To examine whether IFN-γ-treated pOCs were able to resorb bone, the BMMs were seeded on hydroxyapatite-coated 48-well plates and were cultured with M-CSF and RANKL for 3 days to induce their differentiation into pOCs. When pOCs were treated with IFN-γ, fusion of pOCs into multinucleated osteoclasts was induced (Fig. 2A). IFN-γ induces the ability of the cells derived from pOCs to resorb bone (Fig. 2B). These results demonstrate that IFN-γ can induce bone resorption by inducing the fusion of pOCs.

IFN-γ induces the expression of DC-STAMP in pOCs

IFN-γ induces the fusion of pOCs into multinucleated osteoclasts. To examine whether this effect was associated with the induction of fusion-related genes involved in osteoclast fusion, we treated pOCs with IFN-γ and examined their mRNA expression by real time-PCR. IFN-γ significantly induces the expression of DC-STAMP, but it does not induce the expression of Atp6v0d2, β3 intergrin, TRAP, and OSCAR mRNAs (Fig. 3A). To confirm this finding, we treated pOCs with increasing concentrations of IFN-γ. IFN-γ was found to induce DC-STAMP mRNA expression in a dose-dependent manner (Fig. 3B). These results suggest that IFN-γ may regulate the fusion of pOCs through the induction of DC-STAMP.

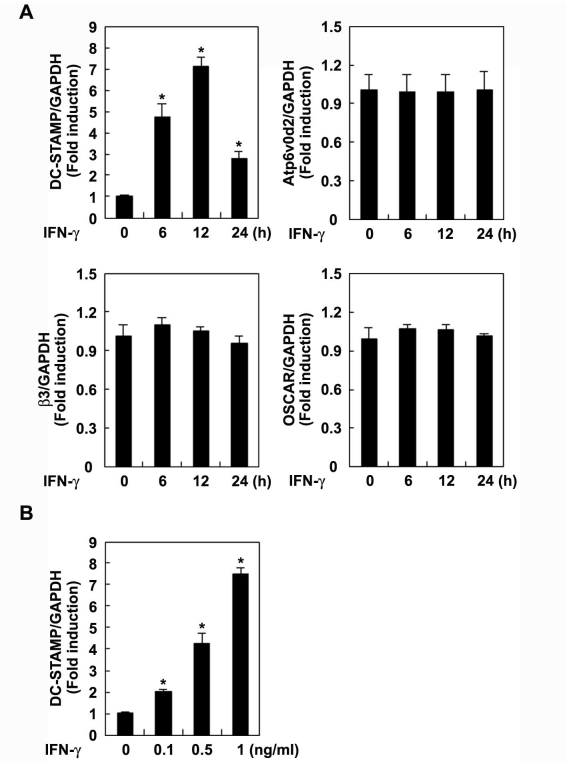

Fig. 3. Expression of DC-STAMP in pOCs treated with IFN-γ. (A) The pOCs were treated with IFN-γ (1 ng/ml) in the presence of M-CSF (20 ng/ml) for the indicated times. Total RNA was extracted, and mRNA expression was analyzed by real time-PCR. Asterisks indicate $P < 0.01$ versus control (without IFN-γ). (B) The pOCs were treated with increasing concentrations of IFN-γ in the presence of M-CSF (20 ng/ml) for 6 h. Total RNA was extracted and analyzed by real time-PCR. Asterisks indicate $P < 0.01$ versus control (without IFN-γ).

IFN-γ induces the expression of c-Fos and NFATc1 in pOCs

The expression of DC-STAMP during osteoclast differentiation is important for the fusion of pOCs into multinuclear osteoclasts (19). NFATc1 can bind to the DC-STAMP promoter and induce the expression of DC-STAMP (15). In addition, c-Fos expression is required for NFATc1 expression (10). Therefore, to determine the effect of IFN-γ in the expression of c-Fos and NFATc1, we stimulated the pOCs with IFN-γ. IFN-γ greatly induces the expression of c-Fos and NFATc1 in pOCs (Fig. 4A). To confirm this finding, we examined the effect of IFN-γ on the expression of c-Fos and NFATc1 proteins. IFN-γ greatly induces the expression of c-Fos and NFATc1 proteins (Fig. 4B). Next, we examined whether the expression of DC-STAMP induced by IFN-γ was inhibited in the presence of the calcineurin inhibitor cyclosporine A (CSA). Inhibition of the NFAT path-

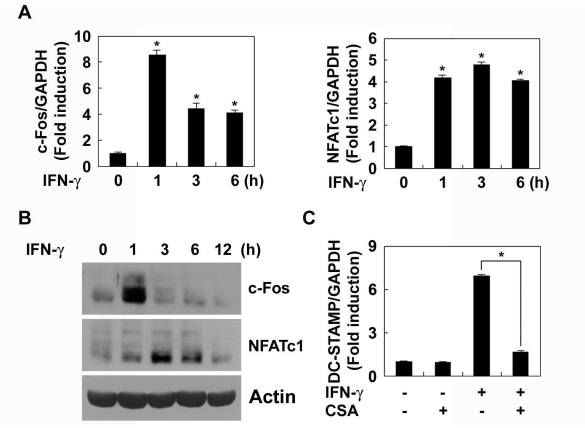

Fig. 4. IFN-γ induces the expression of c-Fos and NFATc1. (A) pOCs were treated with IFN-γ (1 ng/ml) in the presence of M-CSF (20 ng/ml) for the indicated times. Total RNA was extracted, and mRNA expression was analyzed by real time-PCR. Asterisks indicate $P < 0.01$ versus control (without IFN-γ). (B) The pOCs were treated with IFN-γ (1 ng/ml) in the presence of M-CSF (20 ng/ml) for the indicated times. Whole cell lysates from pOCs treated with IFN-γ were subjected to western blotting with anti-c-Fos, anti-NFATc1, and anti-actin antibodies. (C) The pOCs were pretreated with CSA (2 μg/ml) for 1 h and further treated with IFN-γ (1 ng/ml) in the presence of M-CSF (20 ng/ml) for 12 h. Total RNA was extracted and analyzed by real time-PCR. An asterisk indicates $P < 0.01$ between indicated groups.

way reduced the expression of DC-STAMP induced by IFN-γ (Fig. 4C). Taken together, our results suggest that IFN-γ may induce DC-STAMP expression by inducing c-Fos and NFATc1 expression in pOCs.

DISCUSSION

Osteoclasts are multinuclear cells that are formed by cell-cell fusion of pOCs. IFN-γ is expressed by activated T cells and it strongly inhibits osteoclast differentiation by the degradation of TRAF6 (23). The binding of RANKL to RANK results in the recruitment of TRAF proteins from the cytoplasm to the cytoplasmic tail of RANK. In particular, the activation of TRAF6 initiates multiple, complex-signaling cascades that lead to the differentiation of osteoclast precursors (14). In this study, we found that IFN-γ stimulates cell-cell fusion of pOCs into multinuclear osteoclasts (Fig. 1). This finding raises the possibility that IFN-γ induces the fusion of pOCs, because IFN-γ does not exert its activity on osteoclast differentiation. To examine this possibility, we cultured BMMs with IFN-γ in the presence of M-CSF and RANKL. As expected, we found that IFN-γ significantly inhibits osteoclast differentiation. These results demonstrate that although IFN-γ has a negative effect on osteoclast differentiation, IFN-γ can promote the differentiation of pOCs into multinuclear osteoclasts.

Multinucleated osteoclasts are responsible for the resorption of mineralized bone. Bone resorption is tightly regulated by various cytokines and hormones that exert their effect on osteoclasts (4). Recently, Sato et al. reported that IL-4 induces multinucleation of BMMs treated with RANKL. However, IL-4-stimulated multinuclear osteoclasts were only weakly stained by TRAP and they did not resorb bone (25). These results suggest that although IL-4 induces the multinucleation of pOCs, it does not induce bone resorption. Therefore, it is thought that if multinucleated osteoclasts induced by IFN-γ do not resorb bone, then the fusion of pOCs caused by IFN-γ will be futile. In this study, IFN-γ-treated cells typically formed TRAP-positive osteoclasts that resorb bone (Fig. 1 and 2). These results demonstrate that IFN-γ can induce bone resorption as well as differentiation into multinuclear osteoclasts.

DC-STAMP is a seven-transmembrane protein that was originally identified in dendritic cells (26). It has been reported that GM-CSF significantly induces pOC fusion by inducing DC-STAMP expression (21). This finding is consistent with ours, in which only DC-STAMP expression is significantly increased in the pOCs treated with IFN-γ; however, a number of studies have reported the role of fusion-related molecules in the multinucleation of pOCs (16-19). These findings suggest that IFN-γ may regulate pOC fusion through DC-STAMP expression.

NFATc1 was originally identified in T cells and it plays an essential role in osteoclast differentiation (27). Recently, Kim et al. reported that NFATc1 plays a critical role in pOC fusion via the induction of Atp6v0d2 and DC-STAMP (19). Therefore, to examine whether IFN-γ can induce NFATc1 expression, we stimulated pOCs with IFN-γ. The expression of NFATc1 was greatly increased in the pOCs treated with IFN-γ. Furthermore, c-Fos is known to be responsible for the expression of NFATc1 (10). As expected, IFN-γ significantly induces c-Fos expression in pOCs. Taken together, our results suggest that IFN-γ may regulate the expression of NFATc1 by inducing c-Fos expression in pOCs.

In conclusion, IFN-γ mediates the fusion of pOCs into multinucleated osteoclasts by inducing DC-STAMP expression, which, in turn, is regulated by NFATc1 expression via the induction of c-Fos. Although IFN-γ significantly suppresses osteoclast differentiation, it is enriched at sites of inflammation and autoimmune diseases such as rheumatoid arthritis (28). In addition, IFN-γ plays an important role in macrophage activation, inflammation, and immunity by activation of the IFN-γ-inducible genes (29). In this study, we found that IFN-γ promotes cell-cell fusion into bone-resorbing osteoclasts. Thus, IFN-γ may contribute to the progression of bone erosion in pathologic or inflammatory conditions.

MATERIALS AND METHODS

Reagents and antibodies

α-minimum essential medium (α-MEM) and fetal bovine serum (FBS) were obtained from GIBCO/Invitrogen (Carlsbad, CA, USA). Mouse IFN-γ was purchased from R&D Systems (Minneapolis, MN, USA). Antibodies specific for c-Fos and NFATc1 came from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-actin antibody was acquired from Sigma-Aldrich (St. Louis, MO, USA). Hydroxyapatite-coated plates were obtained from Osteogenic Core Technologies (Cheonan, Korea). Cyclosporine A (CSA) was purchased from Calbiochem (La Jolla, CA, USA).

Preparation of bone marrow-derived macrophages (BMMs)

Bone marrow cells (BMCs) were collected from 5-week-old male ICR mice as described previously (21). The BMCs were suspended in α-MEM containing 10% FBS and antibiotics, and were cultured with M-CSF (10 ng/ml) for 1 day. Non-adherent cells were harvested, seeded on 90-mm petri dishes, and then cultured for 3 days in the presence of M-CSF (50 ng/ml). The adherent cells were used as BMMs.

pOC preparation and fusion assay

The BMMs were seeded in 48-well plates at a density of 35,000 cells/well and cultured with M-CSF (20 ng/ml) and RANKL (50 ng/ml) for 3 days to obtain the osteoclasts and used as pOCs. To examine the effect of IFN-γ on pOC fusion, we treated the pOCs with increasing concentrations of IFN-γ in the presence of M-CSF (20 ng/ml) and further incubated the pOCs for 12 h.

TRAP stain

The cultured cells were fixed with 3.7% formalin for 5 min

and then treated with 0.1% Triton X-100 for 5 min. The cells were stained with TRAP solution (Sigma). TRAP-positive cells were counted as multinucleated osteoclasts (nuclear ≥ 5) or osteoclasts (nuclear ≤ 4).

RNA isolation and real time-PCR

Total RNA was extracted from the cells by using the Qiazol reagent (Qiagen, Carlsbad, CA, USA) according to the manufacturer's protocol. 1 μg of RNA was reverse transcribed using dNTPs, oligo(dT) primers, buffer, dithiothreitol (DTT), RNase inhibitor, and Superscript II reverse transcriptase (Invitrogen) in a 20-μl reaction mixture. The mRNA levels were examined by real-time PCR analysis using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The following PCR primers were used: c-Fos forward, 5'-CTGGTGCAGCCCACTCTGGTC-3' and c-Fos reverse, 5'-CTTTCAGCAGATTGGCAATCTC-3'; NFATc1 forward, 5'-CTTCAGCTGGAGGACACCCC-3' and NFATc1 reverse, 5'-GGAAGACCAGCCTCACCCTG-3'; Atp6v0d2 forward, 5'-ATGGGGCCTTGCAAAAGAAA-3' and Atp6v0d2 reverse, 5'-GCTAACAACCGCAACCCCTC-3'; DC-STAMP forward, 5'-GCAAGGAACCCAAGGAGTCG-3' and DC-STAMP reverse, 5'-CAGTTGGCCCAGAAAGAGGG-3'; and GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3' and GAPDH reverse, 5'-TCCACCACCCTGTTGCTGTA-3'.

Western blotting

The cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, and 1% deoxycholate) containing phenylmethylsulfonyl fluoride (PMSF). Whole cell lysates were cleared by centrifugation at 13,000 rpm for 20 min. Following centrifugation, the supernatant was measured by using the DC protein assay kit (Bio-Rad, USA) according to the manufacturer's protocol. Equal amounts of proteins were suspended in a sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). After SDS-PAGE, the gels were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) for 120 min. The transferred membranes were blocked with 5% skim milk in TBST buffer and were incubated overnight with primary antibodies. Next, the membrane was washed in TBST. After washing, the membrane was incubated for 2 h with an appropriate secondary antibody. The target proteins were visualized using an enhanced chemiluminescence solution.

Bone resorption assay

The BMMs were seeded on hydroxyapatite-coated 48-well plates at a density of 100,000 cells/well and further cultured for 3 days with M-CSF (20 ng/ml) and RANKL (50 ng/ml) to induce the formation of pOCs. After 3 days, the pOCs were stimulated for 12 h with or without IFN-γ (1 ng/ml) in the presence of M-CSF (20 ng/ml). The cells were fixed with 3.7% formalin, permeabilized with 0.1% Triton X-100, and stained with TRAP (Sigma). $TRAP^+$ MNCs containing 5 or more nuclei were counted as osteoclasts. To determine the resorption activity, we removed the stained cells from the hydroxyapatite-coated plates, and the plates were photographed under a light microscope (Olympus, Tokyo, Japan). Quantification of the area of resorption was performed by using the Image Pro-plus program, version 4.0.

Statistical analysis

The quantitative results are expressed as mean ± S.D. All statistical analyses were performed by using the Student's *t* test.

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