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Human osteoclast maturation from bone marrow cells co-cultured with osteoblast from ethmoid sinus*

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SUMMARY

In chronic sinusitis, although the pathogenesis in the sinus mucosa has been widely investigated, the pathogenesis in the underlying bone remains poorly understood. As a first step in investigating the pathogenesis in sinus bone, sinus-bone models should be constructed by co-culturing of osteoblasts and osteoclasts in a sinus. However, human osteoclast cell lines derived from a sinus have not been established. Based on previous methods of another osteoclast culture, we hypothesized that human osteoclasts in sinuses could be established in vitro by co-culture with human osteoblasts in the sinus. The aim of this study was to differentiate human bone marrow cells into osteoclasts in a sinus, which is identified by the formation of absorption lacuna and positive cytochemical staining for tartrate-resistant acid phosphatase (TRAP). Differentiation was induced in the co-culture system by treatment with medium containing 1.25.$(OH)_2D_3$, G-CSF and dexamethasone. Thirty days after culturing, human bone marrow cells were differentiated to a hypertrophic state, as evidenced by their sizes and the demonstration of osteoclast-specific abilities. Our results indicate that sinus-osteoclasts can be cultured from human bone marrow cells, and that osteoclasts in a sinus may be a useful model for investigating sinus-bone remodeling, especially in terms of bone resorption, in the sinus system.

Key words: human bone marrow cell, osteoblast, co-culture, maturation, osteoclast

INTRODUCTION

Osteoblasts (or osteoblast-like cells) were the first hard skeletal tissue cells to be successfully cultured *in vitro* on a large scale. The various roles that these cells play in bone metabolism have since been clearly demonstrated. It has proven much more difficult to establish methods for culturing osteoclasts on a large scale; thus, the detailed roles of osteoclasts in the mechanisms of remodeling remain to be clarified. Recently, methods for culturing osteoclasts on a large scale have been established by their co-culture with splenic cells and osteoblasts (or stromal cells), and it has been revealed that osteoclasts play essential roles in bone remodeling. However, no method of co-culturing osteoclasts with bone marrow cells and osteoblasts in the sinus has been established, and thus the sinus-specific activities of osteoclasts have scarcely been investigated.

The aim of this study was to establish a method for large-scale production of osteoclasts in the sinus by co-culturing them with human bone marrow cells from normal patients and osteoblasts in the sinus obtained from human ethmoidal bones. To confirm whether or not osteoclasts were successfully cultured, we investigated various osteoclast-specific abilities in our co-culture system, such as resorption lacuna formation and positive cytochemical staining for tartrate-resistant acid phosphatase (TRAP).

MATERIALS AND METHODS

Osteoblast culture

Osteoblast culturing was performed according to the explant-outgrowth cell culture technique reported by Berensford et al. (1984). Ethmoidal sinus bones were obtained from patients with chronic sinusitis at the time of sinus surgery. Informed consent was obtained from all individuals included in this study. Ethmoidal sinus bones were washed extensively in sterile PBS (phosphate-buffered saline, pH 7.2) to remove blood and debris; the mucosa and periosteum attached to the bone were mechanically removed. The bones were dissected into 1-3 mm^2 pieces and the specimens were harvested as explants onto poly-L-lysine-coated 35 mm cell culture dishes (Asahi Techno Glass, Tokyo, Japan). The cultures were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Sigma,

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St. Louis, MO), 200 units/ml penicillin, 200 μg/ml streptomycin (Meiji Seika, Tokyo, Japan), and 5 μg/ml fungizone (Bristol-Myers Squibb, Tokyo, Japan) and placed in a humidified atmosphere consisting of 95% air and 5% CO_2 at 37°C. The medium was changed every 3-4 days. When the explant-outgrowth cell culture reached the confluent stage, cells were passaged by using 0.25% trypsin and 0.05M ethlenediaminetetraacetic acid (EDTA) (Sigma) in sterile PBS.

Osteoclast differentiation

In order to investigate the recurrence of malignant lymphoma, bone marrow cells were obtained from normal patients (n=3) who had previously been cured of this disease. Informed consent was also obtained from all individuals included in this part of the study. The bone marrow cells were purified by density gradient centrifugation using Separate L (Mutou, Tokyo, Japan). A layer of monoblasts was collected and the cells ($1x10^4$ cells/well) were seeded onto third-passage ethmoidal osteoblasts (n=3) that had been seeded in 96-well tissue culture plates at $1x10^4$ cells/well approximately 24 h earlier.

The cultures were maintained for 30 days in the differentiating medium, which contained α-MEM (ICN Biomedicals, Bryan, OH), 10% FBS supplemented with 10 nM $1,25,(OH)_2D_3$ (Calbiochem, San Diego, CA) and 100 μg/ml G-CSF (TECHNE, Minneapolis, MN), in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. 200 units/ml penicillin, 200 μg/ml streptomycin, and 5 μg/ml fungizone were also added to the medium, which was changed every 3-4 days.

Electron Microscopy

To simulate actual osteoclasts on sinus bones, osteoblasts were cultured for 30 days on ivory slices (0.6 cm diameter, one slice per well) prepared with EXACT BS-3000 at the osteoblast-culture stage by the co-culture method.

1) Scanning electron microscopy (SEM)

The ivory slices were then fixed with 1% glutaraldehyde and an equivalent volume of 1% formaldehyde in 0.1M-phosphate buffer, pH 7.0, for 1 h and were rinsed extensively in phosphate buffer for 30 min. After the fixation, the slices were immersed in 2% tannic acid for 2 h and then extensively rinsed in phosphate buffer for 6 h. For post-fixation, the slices were treated with 1% OsO_4 (0.1M phosphate buffer, pH 7.0) for 1 h. Following dehydration with graded ethanols, the slices were transferred to t-butyl alcohol three times and thereafter freeze-dried with an EIKO ID-2 freeze drier (EIKO, Tokyo, Japan) and sputter-coated with platinum to about 50 Å thickness for scanning electron microscopic observation. A Hitachi S-800 scanning electron microscope with an acceleration voltage of 25 kV was used for observation and photography.

2) Transmission electron microscope (TEM)

The ivory slices were then fixed with 1% glutaraldehyde and an equivalent volume of 1% formaldehyde in 0.1M-phosphate buffer, pH 7.0, for 1 h and were rinsed extensively in phosphate buffer for 30 min.

After fixation, the slices were immersed in 2% tannic acid for 2h and rinsed extensively in phosphate buffer for 6 h. For post-fixation, the slices were treated with 1% OsO_4 (0.1M phosphate buffer, pH 7.0) for 1 h. Following fixation, the slices were stained in 1% uranyl acetate for 40 min and decalcified with 10% EDTA. All specimens were then rinsed three times in 10% saccharose, dehydrated in an ascending series of ethanol, soaked in propylene oxide, and embedded in epoxy resin. Ultrathin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. They were observed with a JEM-100S electron microscope (JEM, Tokyo, Japan) at 80 kV.

Assessment of resorption lacuna

To assess bone resorption by osteoclasts, osteoblasts were also cultured on ivory slices (0.6 cm diameter, one slice per well) at the osteoblast-culture stage in the co-culture method. After 30 days of incubation, cells were removed from the ivory slices by sonication for 30 sec at 30 watts output using a sonic processor for the assessment of bone resorption. For visualization of bone resorption lacuna by scanning electron microscopy, the cell-visualization method 1) (SEM) was also used.

Tartrate-resistant acid phosphatase (TRAP) staining

Cytochemical staining for TRAP, which is also specific to osteoclasts, was carried out. Cells were co-cultured in 96-well plates without ivory slices for 30 days in the differentiating medium. The co-cultured cells were fixed with 2.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 30 min. TRAP staining was performed according to the modified Baroukh's methods (Baroukh et al., 2000). The co-cultured cells were incubated for 60 min at 37°C in a prewarmed moisture chamber with the incubation medium, which contained hexazotized pararosaniline (Sigma), naphthol AS-BI phosphate (Sigma) and L(+)-tartaric acid (Sigma). Data were corrected for the number of TRAP-positive cells in each well.

RESULTS

Scanning electron microscopy (SEM)

We first investigated the differentiated monoblasts by SEM. Based on the discovery of resorption lacunae on the ivory slices that had been stripped of cells (Figure 1), and on the irregular margins of bone resorption lacuna around the non-spindle-like cells in slices that had not been stripped of cells (Figure 2), some of the co-cultured cells must have had the ability to resorb bone.

All the bone-resorptive cells were identified as oval-to-round shaped cells on the slices; osteoblasts, which are spindle-shaped, were identified on the same slices (Figure 3). Since previous reports also demonstrate that osteoclasts are formed from hematopoietic precursors via plasma membrane fusion (Sato et al., 1998), this study suggests that the bone-resorptive cells were differentiated from the monoblasts.

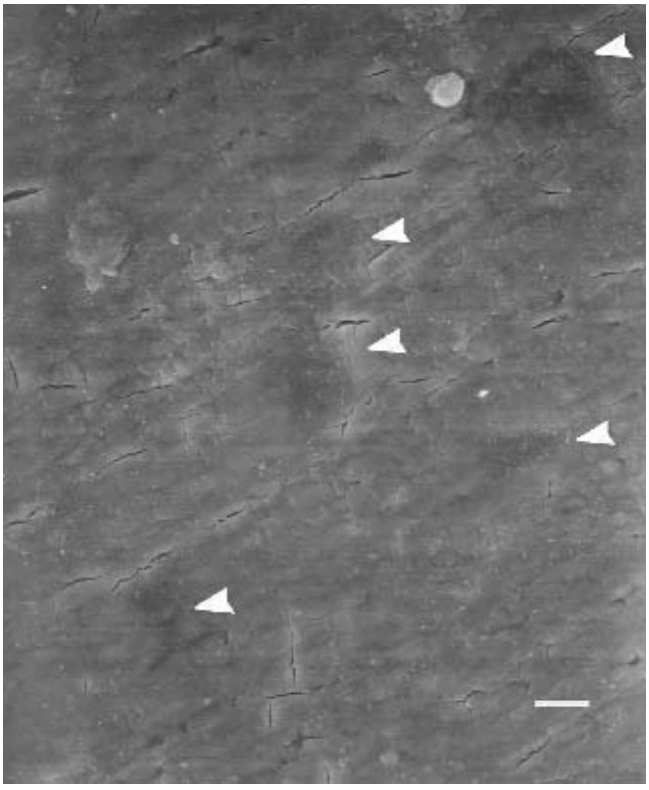

Figure 1. Formation of resorption lacunae (arrowheads) on the ivory slices that had been stripped of cells. Bar = 100 mm.

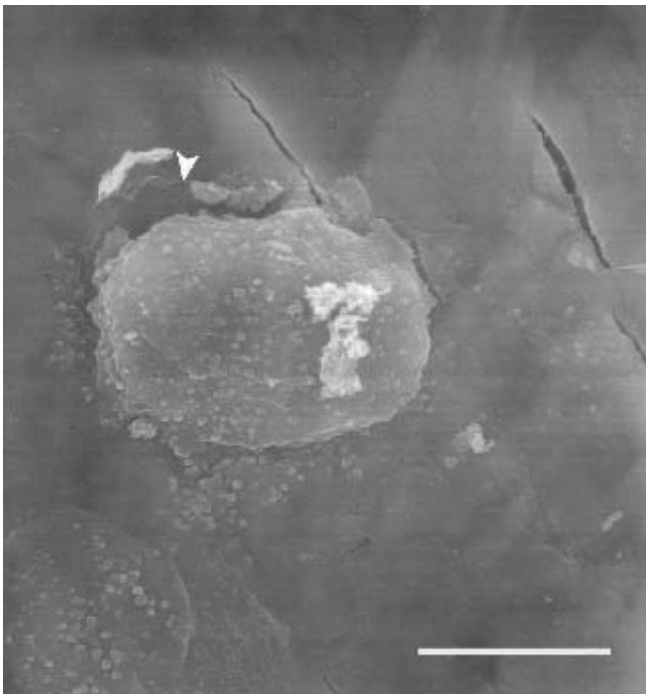

Figure 2. Irregular margins of bone resorption lacuna (arrowhead) around the non spindle-like cells in slices that had not been stripped of cells. Bar = 100 mm.

Transmission electron microscopy (TEM)

We next investigated the bone contact points of the bone-resorptive cells by TEM. In some cultured cells, a construction similar

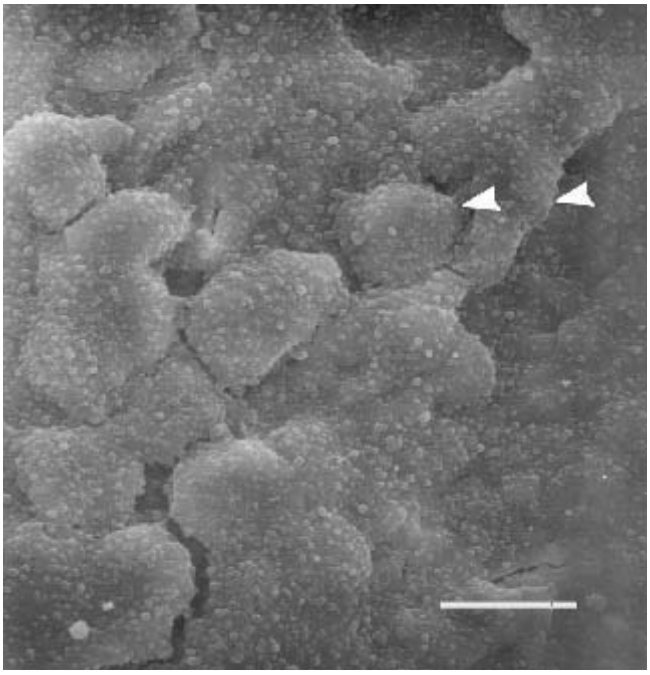

Figure 3. Osteoclast (left arrowhead) and spindle-like cells (right arrowhead) in slices that had not been stripped of cells. Spindle-like cells, which are osteoblasts, were found to be in contact with osteoclasts. Bar = 100 mm.

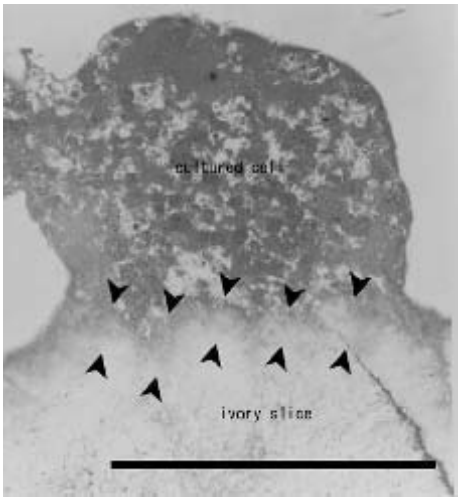

Figure 4. A construction similar to *Ruffled border* in the cell membranes in contact with the ivory slice (arrowheads). Bar = 10 mm.

to *Ruffled border*, which has been recognized as an osteoclast-specific figure and also as an activated bone-resorptive membrane structure, was identified in the cell membranes in contact with the ivory slice (Figure 4). Furthermore, several sizes of vacuoles were also identified in their cytoplasm. Previous reports have demonstrated that the vacuoles in small osteoclasts are generally small and round, while those in large osteoclasts are generally large and ovoid, oblong or polymorphic in shape (Kawamata, 1992); hence, these cultured cells likely consisted of several types of osteoclasts differentiated from monoblasts.

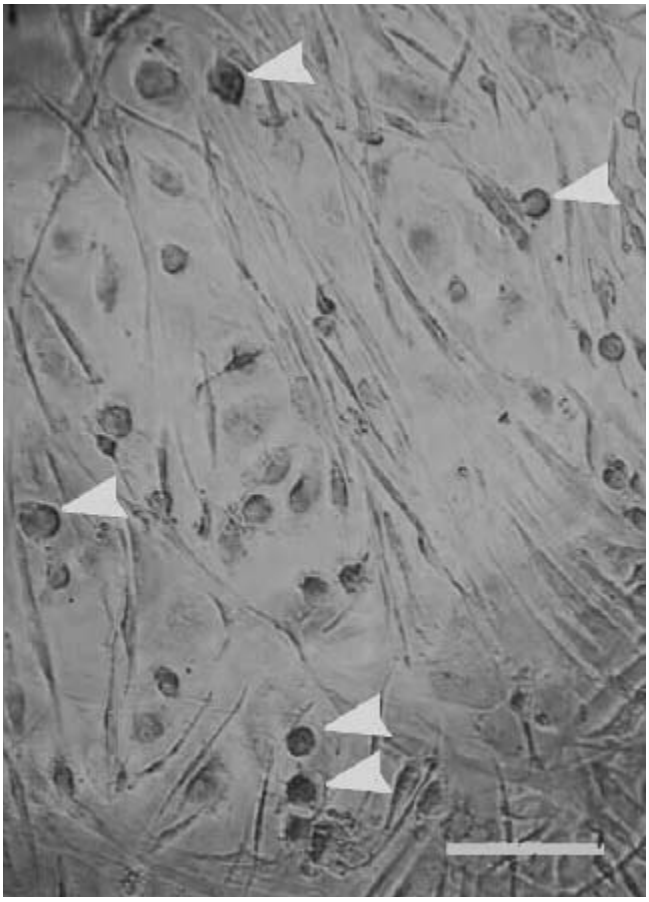

Figure 5. In TRAP-specific staining, the non-spindle-like cells (arrowhead) were highly TRAP-positive, while the osteoblasts were TRAP-negative. The TRAP-positive cells were either the same size as the monoblasts or larger. In TRAP-positive cells, the cells were almost all mononucleated. Bar = 100 mm.

TRAP staining

In TRAP-specific staining, the non-spindle-like cells were highly TRAP-positive, while the osteoblasts were TRAP-negative (Figure 5). The TRAP-positive cells were either the same size as the monoblasts or larger. In TRAP-positive cells, the cells were almost all mononucleated (99.8-100%), indicating immature osteoclasts, while multinucleated cells indicating mature osteoclasts were hardly identified (0-0.2%). The numbers of TRAP-positive cells differed according to the co-cultured osteoblasts (data not shown), ranging from approximately 50 cells/well up to approximately 2200 cells/well.

DISCUSSION

Osteoclast differentiation/proliferation and bone resorption activity have been shown to be controlled mainly by osteoblasts or stromal cells. These cells control osteoclast differentiation/proliferation and bone resorption activity by expressing several cytokines such as Interleukine-1 (IL-1β), IL-6, IL-8, Tumor necrosis factor (TNF-α), Macrophage colony-stimulating factor (M-CSF) and/or specific proteins such as receptor activator of nuclear factor (NF)-κB ligand (RANKL) and the osteoclast differentiation inhibitory factor/osteoprotegerin (OPG). Previous reports have demonstrated that peripheral blood monocytes are capable of differentiating into osteoclasts when co-cultured with stromal cells (Udagawa et al., 1990; Fujikawa et al., 1996a), and synovial macrophages co-cultured with stromal cells are also capable of differentiating into osteoclasts (Fujikawa et al., 1996b). In discussing the effects of cytokines on the proliferation and activation of osteoclasts, Hofbauer et al. (1999) reported that IL-1β and TNF-α stimulate a steady-state mRNA level of osteoprotegerin ligand, which is a critical factor for osteoclastogenesis in various human osteoblastic lineage cells. The osteoblastic cells also produce large amounts of IL-8 in response to IL-1β and TNF-α. IL-8 may be involved in the recruitment and development of bone marrow stromal cells, in osteoclast-osteoblast cell-cell communication, and in the modulation of the microenvironment of the bone and the bone marrow (Chaudhary et al., 1994). Additionally, colony-stimulating factor-1, which is another cytokine released by osteoblasts, is sufficient to support direct osteoclast-formation *in vitro* (Gang-Qing et al., 1998).

Therefore, it considered that *in vitro* co-culturing methods with osteoblasts/stem cells as supporting cells for osteoclasts, and monoblasts as progenitor cells in osteoclasts, were able to simulate the natural environment of human/animal bone surfaces *in vivo*. In previous studies, however, co-cultured human osteoblasts have only been obtained from non-rhinologic sectors of human/animal bones, such as femoral and tibial bones (mesoderm origin) and calvaria and mandibular bones (desmocranium and splanchnocranium origin in neural crest) (Sperber, 1989). Bones of neural crest origin, which include most facial bones, differed from bones of mesodermal origin in terms of their proliferation and maturation pathways *in vivo*, proceeding either by endochondral ossification or by intramembranous ossification. These *in vivo* differences could be clearly recognized in osteodystrophys, such as in van der Hoeve's syndrome (abnormal formation in whole bones), Engelmann syndrome (in cranial base, arms and legs), Cleidcranial dysostosis (in clavicle, calvaria and pubic bones), Ribbing syndrome (in arms and legs) and Idiopathic osteoporosis (in whole bones without cranial bones) (Rubin, 1964). These differences also indicate that bones *in vivo* are distinguished in their proliferation and maturation pathways into three groups: calvaria bones, cranial base, and arms and legs. However, osteoblasts from the cranial base (chondrocranium origin in neural crest), which is constructed of ethmoid and sphenoid bones, have not yet been definitively established. Thus, to differentiate monoblasts into osteoclasts in the sinus, it would seem that co-culturing with sinus osteoblasts was indispensable and that the osteoclasts co-cultured with sinus osteoblasts are different from another osteoclasts.

Co-cultured cells expressed all specific features of osteoclasts in this study; thus, co-cultured cells could be identified as

osteoclasts. Further, our results suggest that the osteoclasts cultured here were different from those produced by co-culturing with other osteoblasts in a previous report (multinucleated cells: 620 cells/spleen cells: $2.5x10^5$ cells) (Sato et al., 1998), in that the cultured cells in the present study were almost all mononucleated. A previous report demonstrated that the differentiation pathways of osteoclast progenitors are mainly regulated by three molecules expressed by osteoblasts, which are the osteoclast differentiation-induced factors M-CSF and RANKL and the osteoclast differentiation inhibitory factor OPG (Suda et al., 1999). It is believed that osteoblasts in the sinus express several molecules such as IL-1β, IL-6, IL-8, TNF-α, M-CSF, RANKL and OPG in specific quantities that differ from those of other osteoblasts, and different expressions of such molecules might lead osteoclasts in the sinus to express sinus-specific abilities in osteoclastgenesis. Therefore, these results additionally show that the osteoclasts produced here were the first cultured osteoclasts to demonstrate sinus-specific abilities.

Chronic sinusitis is often associated with bone remodeling, such as that caused by bone thickening and/or sclerosis, and can be detected by plain radiography or CT scan (Proops, 1983; Unger et al., 1984). To investigate the reasons for such sinus bone remodeling, monoblasts co-cultured with sinus osteoblasts in simulated sinus environments might become possible tools of investigation into sinus-bone remodeling, especially in terms of bone resorption. Our large-scale culturing methods for osteoclasts in sinus might prove useful in future studies of possible causal links in chronic sinusitis between the pathology and physiology of continuous bone remodeling, such as bone thickening or sclerosis and adjacent bone structures. Clearly, osteoclasts play a central role in the bone remodeling process, especially through bone resorption.

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