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Osteoblast Maturation Suppressed Osteoclastogenesis in Coculture with Bone Marrow Cells

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The analysis of co-culture system using osteoblast and bone marrow indicated that the mineralized osteoblast decreased osteoclast formation. This finding was an incentive to better investigate the relation of osteoblast development and osteoclastogenesis. The expression of osteoclast differentiation factor (ODF/RANKL) mRNA and protein dramatically decreased. Alternatively, macropharge colony stimulation factor (M-CSF/CSF-1) transcription and protein secreted in media slightly decreased as the development of osteoblast. On the other hands, mRNA expression and the secretion to the culture medium of osteoclastogenesis inhibitory factor (OPG/OCIF) didn't significantly change depending on osteoblast differentiation. We conclude that osteoblast development might suppress osteoclastogenesis especially with the decrease of ODF/RANKL. © 2000 Academic Press

Key Words: **osteoclastogenesis; osteoblast maturation; co-culture system; ODF/RANKL; OPG/OCIF; M-CSF/CSF-1.**

The development and remodeling of bone tissue entails complex, stringently regulated cell growth and differentiation, with the formation of mineralized bone matrix by osteoblasts coupled to the resorption of that matrix by osteoblast. Recent studies indicated that osteoclast are induced in response to many hormones and cytokines such as parathyroid hormone (PTH), 1α,25-dihydroxyvitamine D_3 (1α,25$(OH)_2D_3$), and interleukin-1β (IL-1β), IL-6, IL-11, and tumor necrosis factor-α (TNF-α) in co-culture system of mouse hemopoietic cells and osteoblasts/stromal cells (1–5). Previously, the experiment with this co-culture system provided that the formed tartrate-resistant acid phosphatase (TRAP), a specific marker for active osteoclasts and their progenitors, positive multinuclear cells had pit-forming activity on dentine slices. It has been believed that the cell-to-cell contact of osteoblasts/stromal cells and hemopoietic cells was essential to osteoclastgenesis (6). Subsequently, ODF/RANKL has been identified and cloned, and this soluble form can generate osteoclasts from hemopoietic cells in the absence of supporting osteoblasts/stromal cells (7–9). Experiments on the osteopetrotic *op/op* mouse model demonstrated that M-CSF/CSF-1, a product of osteoblasts/stromal cells, regulates differentiation of osteoclast progenitor into mature osteoclasts (10, 11). This pit-forming activity of osteoclasts was completely inhibited OPG/OCIF. Thus, ODF/RANKL, a membrane-bound molecule in osteoblast, is crucial for osteoclast fomation along with M-CSF/CSF-1, and that the effects are prevented by its soluble receptor, OPG/OCIF.

Osteoblastic cells undergo a process of proliferation and differentiation and then produce small mineralized nodules. It was reported that there were three distinct periods in the osteoblast development. Initially, there is a period of active proliferation during which cell growth-related genes are actively expressed, and maximal levels of type I collagen mRNA are observed. Following the down-regulation of proliferation, a period of matrix maturation occurs, when the alkaline phosphatase (ALPase) gene is maximally expressed and the extracellular matrix is rendered competent for mineralization, the third period of the developmental sequence (12). We have shown previously that the manifestation of osteoclast decreased as the osteoblast development (13). Furthermore, other studies have indicated that the osteoblast of the adult mouse calvarial bone decreased ODF/RANKL expression and didn't readily support osteoclast formation (14).

The present study was aimed at clarifying how the osteoclasts formation were suppressed as osteoblasts maturation. We have undertaken an analysis of M-CSF/CSF-1, OPG/OCIF, as well as ODF/RANKL

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mRNA and protein expression during osteoblast development.

MATERIALS AND METHODS

Culture of osteoblasst and osteoclast-like cells. Osteoblastic MC3T3-E1 cells (E1 cells) were maintained in α-minimal essential medium (α-MEM) (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (F. Hoffmann-La Roche Ltd., Basel, Switzerland). For induction of osteoclasts-like cells, E1 cells and bone marrow cells obtained from the tibiae of C57/Bl6 mice were co-culured in α-MEM containing 10% FBS, $1\alpha,25(OH)_2D_3$ (Sigma Chemical Co., St. Louis, MO) (10^{-8} M) and dexamethasone (Dex; Sigma Chemical Co.) (10^{-7} M) in 100-mm diameter dishes (Falcon Labware, Oxnard, CA). Osteoclast like cells were formed within 6 days in co-culture. The cells were the fixed with 10% formaldehyde in phosphate-buffered saline (PBS), treated with ethanol-acetone (1:1), and stained for tartrate-resistant acid phosphatase (TRAP) as described previously (15).

Medium ALPase activity. The ALPase activity in the cultured medium which removed from dishes at every 3 days and stocked at −20°C was measured periodically. 150 μl of each sample was assayed by the method of Suzuki *et al.* (16), which is a slight modification of the method of Bessey *et al.* (17). After the addition of 250 μl of the Buffer (12.5 μmol sucrose, 25 μmol carbonate buffer (pH 10.35), and 0.5 μmol $MgCl_2$), the reaction mixture was preincubated at 37°C. The reaction was started by the addition of 100 μl of 10 mM *p*-nitrophenyl phosphate at 37°C, stopped with the addition of 1500 μl of 0.6 N NaOH after 30 min, which developed colour. The amount of *p*-nitrophenyl phosphate liberated was measured spectrophotometrically at 420 nm.

AP-1-DNA binding activity. Nuclear extracts were prepared from the cultured E1 cells at appropriate times according to the method of Schreiber *et al.* (18, 19) with minor modification.

The AP-1 consensus oligonucleotide sequence used as a probe for electrophoresis mobility shift assay (EMSA) (5′-CGC TTG ATG AGT CAG CCG GAA-5′, 3′-GCG AAC TAC TCA GTC GGC CTT-3′) was labeled with [γ-^{32}P] ATP (ICN Biochemicals Ins., Costa Mesa, CA) with use of MEGALABELTM (TAKARA Shuzo Co., Ltd., Kyoto, Japan). The DNA-protein binding reaction was performed for 20 min at room temperature in a reaction mixture (20 μl) containing 20 mM Hepes-NaOH (pH 7.9), 2 mM Tris–HCl, 1 mM dithiothreitol, 0.4 mM EDTA, 0.2 mM EGTA, 80 mM NaCl, 2 μg poly (dI-dC), 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, double-stranded oligonucleotide at 20,000 cpm/μl, and nuclear extract (2 μg). Electrophoresis was carried out at 10 V/cm for 2.5 h at room temperature. After electrophoresis, the gel was dried and exposed to X-ray film (Fuji RX, Fuji Photo Film Co., Tokyo, Japan) on two intensifying overnight at −80°C.

Northern blot and RT-PCR analysis. RNA was isolated from E1 cells by using ISOGENE (Nippon Gene Co., Toyama, Japan), as instructed by the manufacturer, and was separated on formaldehyde-agarose gels, transferred to nylon membrane (Geen Screen Plus; E.I. de Pont de Nemous & Co., Boston, MA) and hybridized with ^{32}P labeled probe. cDNA synthesis was performed in 25 μl from 1 μg of total RNA using the Superscript II moloney murine leukemia virus reverse transcriptase and oligo-dT primer (GIBCO BRL, Life Technologies, Inc., Rockville, MD). The amount of 1 μg of the cDNA product was amplified with the AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Inc., Braunchburg, NJ) in 25 μl, and conditions were experimentally assessed for each pair of primers to allow for an analysis in the exponential range. The PCR program was as follows: 35 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and the $MgCl_2$ concentration was 1.5 mM. The following primers were used: 5′-TAT GAT GGA AGG CTC ATG GT-3′ and 5′-TGT CCT GAA CTT TGA AAG CC-3′.

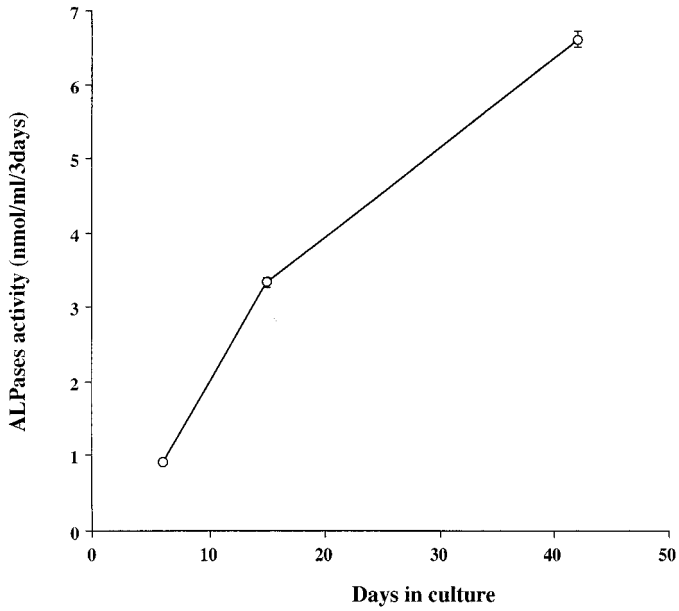

FIG. 1. Changes of ALPase activity of the E1 cells during osteoblast development. Samples were collected at the indicated time and the ALPase activity was assayed as described under Materials and Methods. The horizontal axis represents the number of days after plating. Bars represent means ± SEM from 3 different cultures. $^*P < 0.001$.

Western blot. Before cell harvest, the media were removed from dishes at every 3 days and stocked at −20°C until assay.

Cells were washed twice with ice-cold PBS then lysed on ice with 0.25 M sucrose and transferred to microcentrifuge tube. Protein extracts were separated from the cell debris by centrifugation (15,000*g*, 10 min) at 4°C. Equal amounts of protein lysates (30 μg) or culture mediun (15 μl) were resolved on 10% SDS–PAGE gel, transferred to PVDF membrane (Immobilon Transfer membrane, Millpore Co., Bedford, MA) for Western blotting. The membranes were blocked by overnight incubation at 4°C in TBST buffer (0.1% Tween 20, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4) containing 5% non-fat milk, washed, and subsequently incubated with RANKL (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), OPG (R&D Systems, Inc., Minneapolis, MN) or M-CSF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) atibody for 1 h at room temperature with shaking. The membrane was then washed three times in TBST buffer, incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG (ZYMED Laboratories Inc., South San Francisco, CA), washed in TBST, and visualized by enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

RESULTS

Development of Osteoblastic Cells

To ensure the degree of development of E1 cells, we evaluated ALPase activities in culture medium at each culture period. ALPase activity increased time-dependently (Fig. 1). Likewise, AP-1-DNA binding activity, a marker of cell growth, of developing E1 cell decreased as the progression of E1 cells (Fig. 2). These results indicate that E1 cells matured with progression of culture.

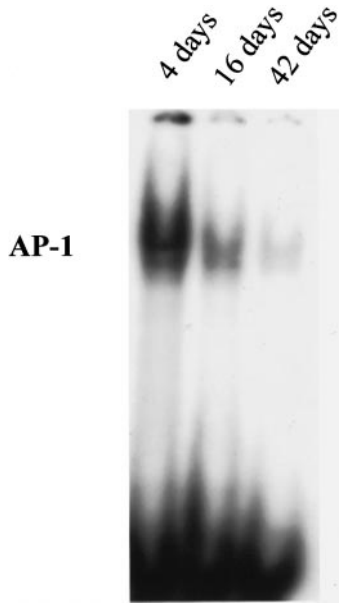

FIG. 2. AP-1 DNA binding activity in developing MC-3T3-E1 cells. EMSA of nuclear extacts from E1 cells cultured at 4, 16, and 42 days after plating.

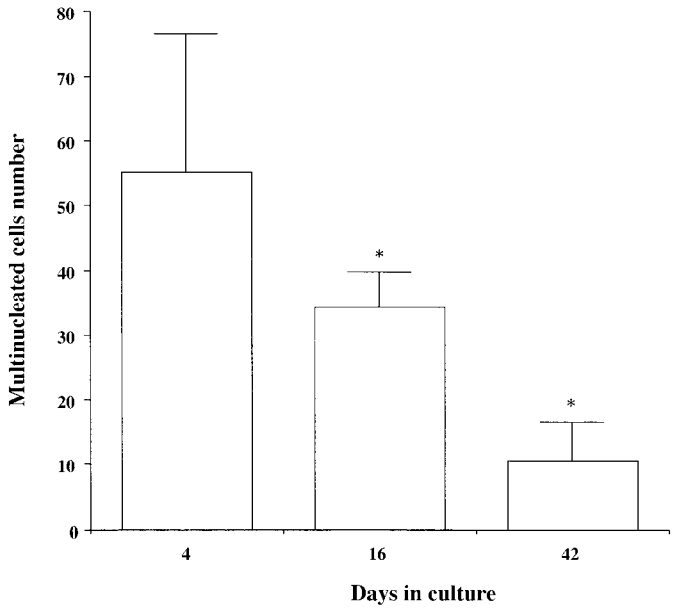

FIG. 3. Time course of change in TRAP-positive MNC formation in co-cultures of MC-3T3-E1 cells and mouse bone marrow cells. The results are expressed as the means ± SEM of three cultures. Bars represent means ± SEM from three different cultures. $*P < 0.05$.

Formation of Osteoclast-like Cells with Developing Osteoblasts

Osteoblastic E1 cells were cultured to confluence, 16 or 42 days in culture, and then co-cultured with bone marrow cells for additional 6 days. Addition of 10^{-8} M $1\alpha,25(OH)_2D_3$ and 10^{-7} M Dex induced TRAP-positive multinucleated cells. TRAP-positive multinucleated cells decreased as progression of osteoblast developmental stage (Fig. 3).

Expression of ODF/RANKL, OPG/OCIF, and M-CSF/CSF-1 mRNA in Osteoblastic Cells

As a further demonstration of the suppression of osteoclast like cells formation, we examined ODF/RANKL, OPG/OCIF, and M-CSF/CSF-1, factors relation to osteoclastogenesis, mRNA expression in E1 cells. The expression of OPG/OCIF gene, which inhibits osteoclast formation, was consistently expressed and remained unchanged with the increase in ALPase activity in cultures (Fig. 4). Yet, ODF/RANKL and M-CSF/CSF-1, which are absolute requirement for osteoclast development, transcripts decreased following of osteoblasts maturation. Moreover, decrease of ODF/RANKL was dramatically whereas M-CSF/CSF-1 decreased slightly (Figs. 4 and 5).

Expression of ODF/RANKL Protein in Differentiating Osteoblasts

ODF/RANKL binds to RANK (receptor activator of NF-κB) on hematopoietic cells and promote differentiation of macrophage/monocyte into osteoclasts with conjunction with M-CSF/CSF-1. Therefore, we investigated ODF/RANKL protein expression using Western blot analysis. ODF/RANKL on cell surface decreased as the cultures progressed into the mineralization stage (Fig. 6).

Secretion of OPG/OCIF and M-CSF/CSF-1 to the Culture Medium

As osteoclastogenesis with co-culture system regulated by the factors secreted from osteoblast as well as

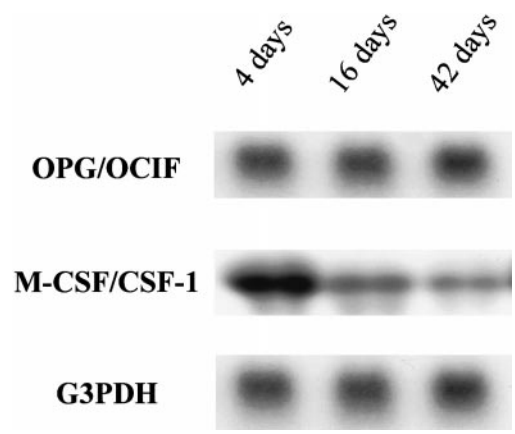

FIG. 4. OPG/OCIF and M-CSF/CSF-1 mRNA expression levels depend on culture time. Northern blots of RNAs isolated from E1 cells cultured at 4, 16, and 42 days after plating. Control hybridization was performed with use of G3PDH probe.

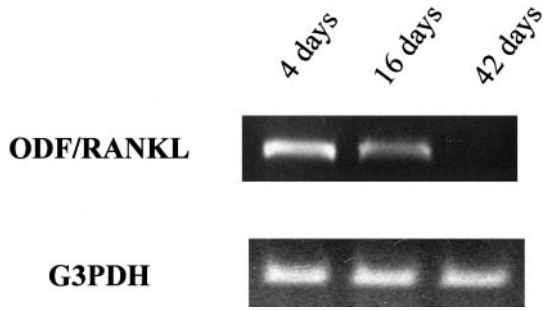

FIG. 5. Expression of ODF/ RANKL mRNA by maturing osteoblastic E1 cells. RT-PCR for ODF/RANKL and G3PDH of RNA isolated from E1 cells cultured at 4, 16, and 42 days after plating.

that of osteoblast cell surface, we examined OPG/ OCIF, which inhibit osteoclast formation and maturation, and M-CSF/CSF-1, essential to osteoclast development, in culture medium. OPG/OCIF in culture medium was detected during all the osteoblast developmental sequences and remained invariable through all osteoblast developmental sequences. Furthermore, M-CSF/CSF-1 secretion slightly declined as progressive osteoblast phenotype development (Fig. 7).

DISCUSSION

Bone remodeling is a complex process coupling bone formation and resorption. The bone tissue contains various types of cells, of which the bone-forming osteoblasts and bone-resorbing osteoclasts are mainly responsible for bone remodering. Osteoblasts are believed to be derived from undifferentiated mesenchymal cells, which produce various bone matrix and cytokines and further differentiate into osteocytes and are embedded in bone. Osteoclasts are believed to be derived hemopoietic origin, and are recruited from hematopoietic tissues such as bone marrow and spleen, and circulating blood to bone. Differentiation of osteoblast occurs in three stages. First, actively proliferating osteoblasts express cell growth-related genes, c-*fos*, c-*jun*, and H4 histone, and genes associated with extracellular matrix biosynthesis, such as type I collagen. Subsequently, at a critical transition point, an integrated series of signals mechanisms mediates cessation of cell division, resulting in the down-regulation of proliferation-specific genes and the initiation of osteoblast phenotype gene expression, such as ALPase. As a result of genes expressed during both the initial proliferative and immediate postproliferative periods, the extracellular matrix is rendered competent for ordered deposition of hydroxyapatite by mature osteoblasts. Extracellular matrix mineralization is initiated at a second transition point, coincident with the up-regulation of mature bone phenotypic genes, including osteocalcin and osteopontin.

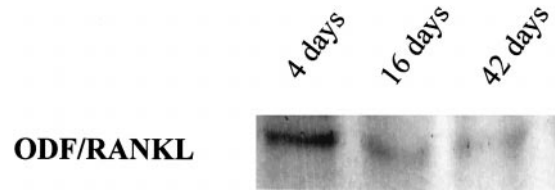

FIG. 6. Secretion of OPG/OCIF and M-CSF/CSF-1 to culture medium from developing E1 cells. Western blots of culture medium removed from dishes every three days after treatment of $1\alpha,25(OH)_2D_3$ and Dex. E1 cells were cultured for 4, 16, and 42 days after plating and then added 10^{-8} M $1\alpha,25(OH)_2D_3$ and 10^{-7} M Dex.

We examined the relation of osteoblast differentiation and osteoclastogenesis in co-culture system. We used E1 cells at three stage, just confluence, 16 and 42 days after plating in co-culture. Osteoblastic E1 cells differentiated with the increasing of ALP activity time-dependently and decreasing of AP-1 binding activity. This result was supported by the reported observation (20). In co-culture of E1 cells and bone marrow cells, the osteoclast formation decreased as osteoblast maturation. It revealed that osteoblast differentiation suppressed osteoclastogenesis. To achieve this suppressive mechanism, we examined the factors which regulate osteoclasts formation. To date it has been reported that some factors, such as ODF/RANKL, OPG/OCIF (21, 22), M-CSF/CSF-1 (23, 24), and IL-6, have been known to express on plasma membrane on osteoblasts or secreted from osteoblasts, and regulate osteoclastogenesis.

First, we assayed these gene expressions in developing osteoblasts by Northern blot and RT-PCR. ODF/ RANKL and M-CSF/CSF-1 mRNA decreased whereas OPG/OCIF remained unchanged. This led us to the possibility that all of these phenomena might impair formation of osteoclasts. We therefore undertook the studies of protein coding these genes.

Second, we assessed the osteoclastogenesis regulatory factors released from osteoblastic E1 cells. Osteoblast maturation decreased M-CSF/CSF-1 release like mRNA expressions. It has been revealed normal secre-

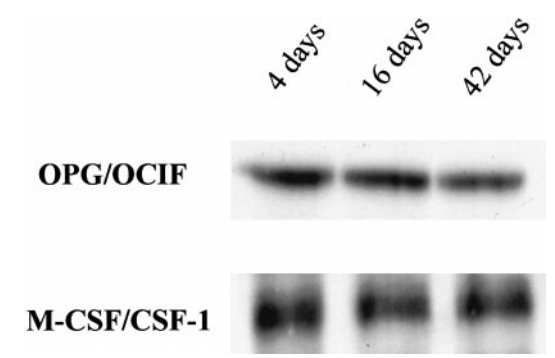

FIG. 7. Expression of ODF/RANKL on the surface of developing E1 cells. Western blots of proteins isolated from e1 cells cultured at 4, 16, and 42 days after plating.

tion of M-CSF/CSF-1 is required for normal osteoclast formation. In the absence of M-CSF/CSF-1, osteoclast generation, osteoclastic bone resorption, and normal marrow cavity formation do not occur (23, 24). When ODF/RANKL binds to RANK (receptor activator of NF-κB) on hemopoietic cells, the signals of osteoclastogenesis mediate in osteoclast precursor. To date two receptors identified for ODF/RANKL are RANK and a secreted receptor, OPG/OCIF, which is thought to behave as a natural decoy receptor to limit biological actions of ODF/RANKL. OPG/OCIF was found to inhibit osteoclast maturation *in vitro*, and when overexpressed in transgenic animals, resulted in osteopetrosis as a result of inhibiting normal octeoclast remodeling of bone. It is tempting to speculate that with osteoblast maturation, ODF/RANKL on osteoblasts decreased, so the binding of RANK on bone marrow cells to ODF/RANKL may decrease and osteoclast formation may be suppressed. The transcription as well as secretion of M-CSF/CSF-1 changed slightly whereas ODF/RANKL decreased dramatically. It is conceivable that M-CSF/CSF-1 may be trivial factor and ODF/RANKL may be main factor in the suppression of osteoclastogenesis with the osteoblast maturation in co-culture system. OPG/OCIF remained unchanged through all culture periods. This fact indicated that OPG/OCIF may have no function to suppress osteoclast formation by osteoblast maturation.

ACKNOWLEDGMENTS

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