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Isolation of a Novel Cytokine from Human Fibroblasts That Specifically Inhibits Osteoclastogenesis

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A factor which inhibits osteoclast-like cell formation was found in the conditioned medium of human embryonic lung fibroblasts, IMR-90. The factor, termed osteoclastogenesis inhibitory factor, OCIF, was purified to homogeneity. OCIF is a heparin-binding basic glycoprotein and has been isolated as a monomer with an apparent molecular weight (Mr) of 60,000 and a homodimer with a Mr of 120,000. The N-terminus of OCIF is blocked and the determination of internal amino acid sequences revealed that OCIF has no homology to known proteins. OCIF inhibited in a dose-dependent manner osteoclastogenesis elicited through three distinct signaling pathways stimulated by 1α,25-dihydroxy vitamin D_3, parathyroid hormone, and interleukin-11, respectively, in a dose range of 1 to 40 ng/ml (IC50 = 4 to 6 ng/ml). OCIF neither inhibits bone resorption by mature osteoclasts nor exerts any other biological activities. These data strongly suggest that OCIF is a novel cytokine which specifically inhibits osteoclastogenesis.

Bone is a dynamic tissue which is morphogenized and maintained by continuous formation and resorption. Multinucleated osteoclasts are the cells primarily responsible for the bone resorption (1, 2). Osteoclasts derive from hematopoietic precursor cells such as colony forming unit-granulocyte macrophage (CFU-GM) or more differentiated monocytic cells (3-5). The precursor cells differentiate into mature osteoclasts at the site of bone resorption under the control of osteotropic hormones, local factors produced in the microenvironment, and osteoblastic stromal cells (6, 7). In vivo studies on op/op mice bearing a mutation in macrophage-colony stimulating factor (M-CSF) gene demonstrated that M-CSF is one of the factors responsible for the differentiation of both osteoclasts and monocytes/macrophages (8-10). Similarly, it has been suggested that interleukin (IL)-6-mediated stimulation of osteoclastogenesis after estrogen loss accelerates bone resorption in postmenopausal osteoporosis (11).

Osteoclast-like cells can be formed in vitro by culturing bone marrow cells or by coculturing spleen cells with osteoblastic stromal cells, in the presence of stimulators of bone resorption such as IL-6, parathyroid hormone (PTH), and 1α,25$(OH)_2D_3$ (12-14). Osteoblastic stromal cells are essential for osteoclastogenesis and involved in osteoclast differentiation by secreting soluble factors and by signaling to osteoclast progenitors through cell-to-cell interaction (14-16). However, the precise differentiation pathway from osteoclast progenitors into mature osteoclasts is still unclear. For the understanding of the processes in which hematopoietic precursors differentiate into mature osteoclasts, identification of factors involved in the processes is essential.

We found a factor, which inhibits osteoclast development from unfractionated bone cells, in the conditioned medium of human fibroblasts. In this report, we describe the purification to homogeneity of a novel cytokine termed osteoclastogenesis inhibitory factor, OCIF.

MATERIALS AND METHODS

Conditioned medium of human fibroblasts. Human embryonic lung fibroblasts, IMR-90 (ATCC, CCL186), were statically cultured in roller bottles (490 cm^2, Corning) each containing 80 g of alumina ceramic pieces (3-5 mm, Toshiba Ceramic, Japan) as the cell-adherence matrices and 500 ml of DMEM supplemented with 5 % new born calf serum and 10 mM HEPES buffer as described previously (17). Using 60 roller bottles, about 30 liters of the conditioned medium was obtained per each cycle of cultivation.

Assay for OCIF activity. OCIF activity in the samples obtained from each purification step was determined by observing the suppres-

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Abbreviations used: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PTH, parathyroid hormone; TRAP, tartaric acid-resistant acid phosphatase; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CT, calcitonin.

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sion of osteoclast-like cell formation. Osteoclast-like cell formation was assessed as described by Takahashi et al. (13) as follows except for the use of 96-well plates in place of 24-wells. Unfractionated mouse bone cells were cultured with 10 nM 1α,25$(OH)_2D_3$ for seven days and stained for tartaric acid-resistant acid phosphatase (TRAP)-positive cells using an acid phosphatase kit (No. 387-A, Sigma). One unit of OCIF was defined as the amount required for 50 % inhibition of TRAP-positive cell formation.

Suppression of osteoclastogenesis by purified OCIF was determined using the following three systems each utilizes distinct signaling pathway to induce osteoclast-like cell formation. In the first system, unfractionated mouse bone cells were cultured in the presence of 1α,25$(OH)_2D_3$ or PTH (13). In the second, ST2 stromal cells (RIKEN CELL BANK, RCB0224, Japan) and mouse spleen cells were cocultured in the presence of 1α,25$(OH)_2D_3$ (14). In the third, preadipocyte MC3T3-G2/PA6 cells (PA6) (RIKEN CELL BANK, RCB 1127, Japan) and mouse spleen cells were cocultured in the presence of IL-11 as follows. PA6 cells and spleen cells from ddy mice (8 week-old) were suspended in α-MEM containing 10 % FBS and 10 ng/ml murine recombinant IL-11 (genzyme) and were inoculated into each well at cell densities of 5×10^3 cells/50μl/well and 1×10^5 cells/50μl/well, respectively. Purified OCIF was serially diluted with the same medium, and 100 μl of the diluted OCIF was added to each well in 96-well microplates. After cultivation at 37°C for seven days in the presence or absence of 0.1 to 100 ng/ml OCIF, osteoclast-like cell formation was assessed by counting TRAP-positive cells, by determining TRAP-activity in the cultured cells, or by analyzing expression of calcitonin (CT) receptor as described below.

Purification of OCIF. Approximately 90 liters of the conditioned medium of human fibroblasts, IMR-90, was filtrated through 0.22 μm membrane filter (hydrophilic Milidisk, 2000 cm^2, Millipore), and was divided into three equal portions. Each 30 liters of the conditioned medium was applied to a heparin Sepharose CL-6B column (5 × 4.1 cm, Pharmacia) equilibrated with 10 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl at a flow rate of 500 ml/hour. After washing the column with the same buffer, proteins were eluted from the column with 10 mM Tris-HCl, pH 7.5, containing 2 M NaCl at a flow rate of 500 ml/hour. The eluate was dialyzed against 20-fold volume of 10 mM Tris-HCl, pH 7.5 at 4°C overnight, and was supplemented with 0.1% CHAPS (Sigma). The eluate was applied to a HiLoad Q/FF column (2.6 × 10 cm, Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.1% CHAPS (Tris-CHAPS buffer) at a flow rate of 4 ml/min and 1000 ml of a non-adsorbent fraction with OCIF activity was obtained. Subsequently, the non-adsorbent fraction was applied to a HiLoad S/HP column (2.6 × 10 cm, Pharmacia) equilibrated with Tris-CHAPS buffer at a flow rate of 4 ml/min. After the column was washed with the same buffer, proteins bound to the column were eluted with a 800 ml linear gradient from 0 to 1 M NaCl at a flow rate of 8 ml/min and fractions (12 ml) were collected. Fractions number 1 to 40 were combined into four pools each composed of ten fractions and tested for OCIF activity. The active pool (Fr. 21-30) was diluted 2-fold with Tris-CHAPS buffer and applied to a heparin 5PW column (0.8 × 7.5 cm, Tosoh, Japan) equilibrated with Tris-CHAPS buffer at a flow rate of 1 ml/min. The column was washed with the same buffer and then developed with a 30 ml linear gradient from 0 to 2.0 M NaCl at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and tested for OCIF activity. The active fractions, Fr. 27-38, were pooled, diluted 10-fold with Tris-CHAPS buffer and applied to a cibacron-blue 5PW column (0.5 × 5 cm, Tosoh) equilibrated with Tris-CHAPS buffer at a flow rate of 1 ml/min. The column was washed with the same buffer and then developed with a 30 ml linear gradient from 0 to 2 M NaCl at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and tested for OCIF activity. The active fractions, Fr. 43-65, were acidified with 25% trifluoroacetic acid (TFA, a final conc. 0.1%), and applied to a reverse-phase C4 column (2.1 × 220 mm, BU-300, Perkin-Elmer) equilibrated with 25% acetonitrile containing 0.1 % TFA. Proteins bound to the column were eluted with a 12 ml linear gradient from 25 to 55% acetonitrile containing 0.1 % TFA at a flow rate of 0.2 ml/min and each protein peak was collected. One hundred μl of each protein fraction was immediately diluted with 900 μl of α-MEM containing 10 % FBS, and was tested for OCIF activity. Protein concentration was determined by the DC protein assay kit (BioRad) using bovine serum albumin (BSA) as a standard protein.

SDS-polyacrylamide gel electrophoresis. SDS-PAGE was performed using the Pharmacia Fast System and PhastGel Separation Media (10 to 15% gradient polyacrylamide gel) according to the manufacturer's protocol.

Internal amino acid sequence. Purified OCIF (about 12 μg) was pyridylethylated under reducing conditions (18) and digested with lysylendopeptidase (Wako Pure Chemical, Japan) (19). The digested sample was supplemented with 0.1% TFA and applied to a reverse-phase C_8 column (RP-300, 2.1×220 mm, Perkin-Elmer) equilibrated with 0.1% TFA. The peptide fragments were eluted from the column with a 14 ml linear gradient from 0 to 50 % acetonitrile containing 0.1 % TFA at a flow rate of 0.2 ml/min, and each peptide peak was collected. Each peptide fragment was subjected to sequence analysis with a PROCISE Protein Sequencer 494 or 492 (Perkin-Elmer).

Assay for TRAP activity. The cultured cells in 96-well plates were fixed with ethanol/acetone as described previously (13) and TRAP activity was determined as follows. One hundred μl of 50 mM citrate buffer, pH 4.5, containing 5.5 mM p-nitrophenol phosphate and 10 mM sodium tartrate was added to each well in the plates. After incubation for 15 min at room temperature, 20 μl of 0.1 N NaOH was added to each well and absorbance at 405 nm was measured using an ImmunoReader NJ-2000 (InterMed).

Assay for calcitonin (CT)-receptor. Unfractionated mouse bone cells or a mixture of mouse spleen cells and ST2 stromal cells was cultured in 96 well-plates in the presence of 1α,25$(OH)_2D_3$ or PTH (13, 14) and was washed with PBS containing 0.1 % BSA. The cells were then incubated with 0.5 nM [^{125}I]-salmon CT (sCT, Amersham) in 100 μl of α-MEM medium containing 0.1% BSA and 20 mM HEPES buffer for one hour at 37°C, in the presence or absence of 400-fold excess concentrations of unlabeled sCT (Cosmo Bio, Japan). The cells were washed with PBS containing 0.1% BSA and lysed in 100 μl of 0.1 N NaOH. The radioactivity in the lysate was determined using a gamma counter (AUTO-GAMMA 5650, PACKARD).

RESULTS

Purification and characterization of OCIF. Major osteoclastogenesis-inhibitory activity in the IMR-90 conditioned medium was bound to a heparin CL-6B column and was eluted with the buffer containing 2 M NaCl. When 1.5 liters of new born calf serum (NBCS), which is equivalent to the amount of NBCS in the IMR-90 conditioned medium, was applied to the same column, no such activity was detected in the heparin-binding fractions. OCIF activity did not bind to a HiLoad Q column indicating that OCIF is a basic protein. OCIF was purified by successive chromatography on HiLoad S (Fig. 1A), heparin 5PW (Fig. 1B), cibacron-blue 5PW (Fig. 1C), and reverse-phase C4 (Fig. 1D) columns. OCIF activity was detected in two peaks, peak-6 and -7 (Fig. 1D). The proteins of the two peaks were analyzed by SDS-PAGE (Fig. 2). Peak-6 gave a single band with an apparent molecular weight (Mr) of 60,000 under both reducing and non-reducing conditions. Peak-7 gave a single band with a Mr of 120,000 under non-reducing conditions, and with a Mr of 60,000 under

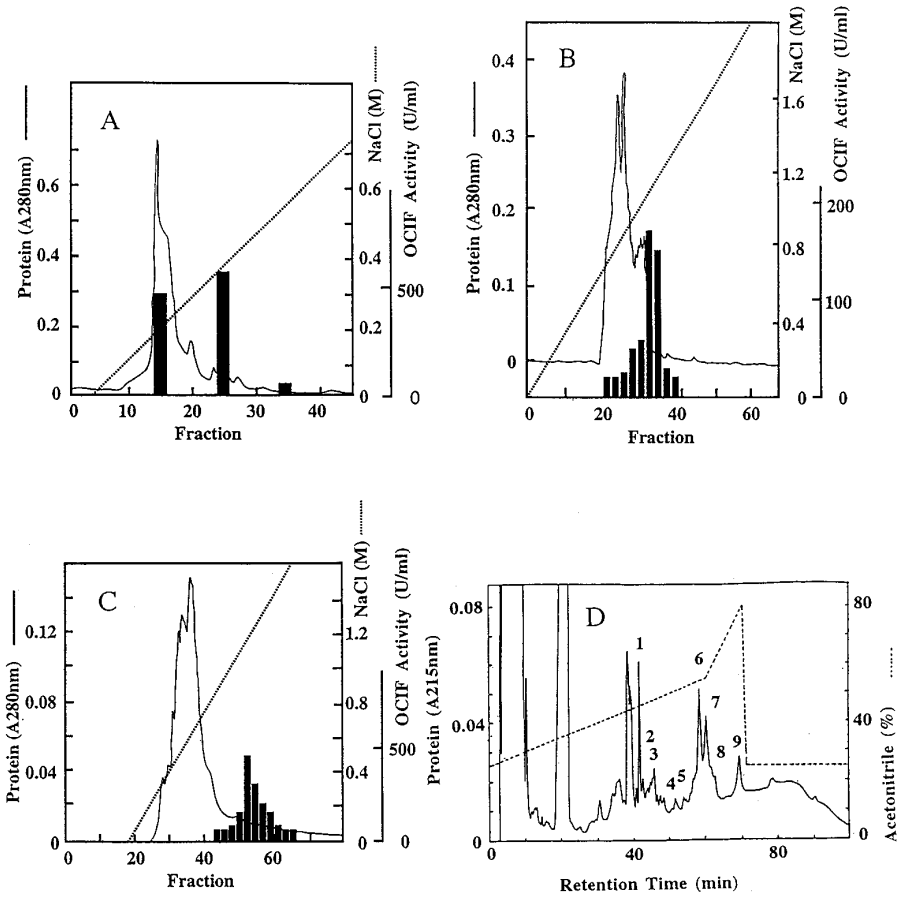

FIG. 1. Purification of OCIF from conditioned medium of human fibroblasts. A, HiLoad S/HP ion-exchange chromatography. B, heparin-5PW affinity chromatography. C, cibacron blue-5PW affinity chromatography. D, reverse-phase C_4 HPLC.

reducing conditions. These results suggested that the protein of peak 7 is a homodimer of that of peak 6. The digestion of each OCIF with N-glycanase under reducing conditions shifted the Mr from 60,000 to 40,000, indicating that OCIF is a glycoprotein with N-linked sugar chains in its molecule (Fig. 2). Determination of N-terminal amino acid sequence of OCIF was not successful, suggesting that N-terminus of OCIF is blocked. To determine internal amino acid sequences of OCIF, the protein of peak-7 was pyridylethylated and digested with lysylendopeptidase. The peptide fragments produced by the digestion were fractionated by a reverse-phase C_8 column-HPLC (Fig. 3) and amino acid sequences of three peptides (P1, P2, and P3) were determined. The sequences were P1, X Y H F P K ; P2, X Q H S X Q E Q Y F Q L X K ; and P3, X I R F L H S F Y M Y (X, not identified). The amino acid sequences of the peptide fragments derived from the peak-6 protein were identical to those of the corresponding peptides derived from the peak-7 protein (data not shown), thereby confirming that the protein of peak-7 is a homodimer-form OCIF. A homology search against protein databases (NBRF-PDB and SWISS-PROT) revealed that OCIF has no homology to known proteins. The amount of protein in each peak was about 30 μg. The monomer- and the homodimer-form OCIF had almost the same specific activity in terms of inhibition of osteoclast-like cell formation as described below. From 90 liters of the conditioned medium, we obtained 60 μg of purified OCIF, representing 550,000-fold purification and 3.6 % yield.

Biological characterization of OCIF. Inhibition of osteoclast-like cell formation by OCIF was tested using three stimulators of osteoclastogenesis each utilizes distinct signaling pathway. Both $1\alpha,25(OH)_2D_3$- and PTH-induced osteoclast-like cell formation from unfractionated mouse bone cells was inhibited by the addition of OCIF to the culture medium in a range of 1 to 40 ng/ml (Fig. 4A). The half inhibition concentration was 4 to 6 ng/ml. OCIF also inhibited osteoclast-like cell formation from mouse spleen cells in two separate experiments in the same dose range. In one experiment, the spleen cells were cocultured with ST2 stromal cells in the presence of $1\alpha,25(OH)_2D_3$ (Fig. 4B). The monomer-form and the homodimer-form OCIF

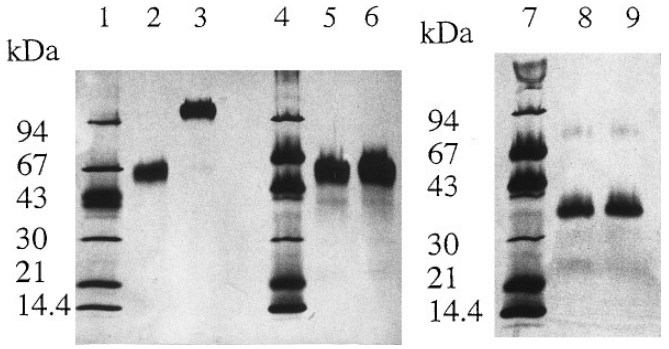

FIG. 2. SDS-PAGE of purified OCIF. Aliquots (30 μl, 300 ng protein) from active fractions, peak-6 and peak-7, from reverse-phase HPLC (Fig. 1D) were dried under vacuum and dissolved in 1.5 μl of 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 2.5% SDS and 0.01% bromophenol blue, and incubated at 37°C overnight in the presence or absence of 5% 2-mercaptoethanol. One μl of each sample was loaded on a polyacrylamide-gradient gel. After electrophoresis, protein bands were visualized with silver stain. Aliquots (200 μl, 2 μg protein) from active fractions, peak-6 and peak-7, from reverse-phase C4 HPLC (Fig. 1D) were dried under vacuum and digested with 0.3 μl of 250 U/ml N-glycanase (genzyme) under reducing conditions according to the manufacturer's protocol and then subjected to SDS-PAGE as described above. Lanes 1, 4, and 7 ; molecular weight standards (Pharmacia) ; lanes 2 and 3, protein of peak-6 and -7, respectively, under non-reducing conditions ; lanes 5 and 6, protein of peak-6 and-7 under reducing conditions ; lanes 8 and 9, protein of peak-6 and -7 digested with N-glycanase under reducing conditions.

gave similar specific activity (Fig. 4B). In the other experiment, the spleen cells were cocultured with PA6 cells in the presence of IL-11 (Fig. 4C). The decrease of TRAP activity coincided with the decline of the binding of CT (Fig. 4B and 4D), indicating that OCIF inhibited osteoclast development. On the other hand, OCIF neither showed any effect on nonspecific esterase-positive macrophages (Fig. 4A) nor inhibited bone resorption (pit formation) by mature rat osteoclasts on ivory

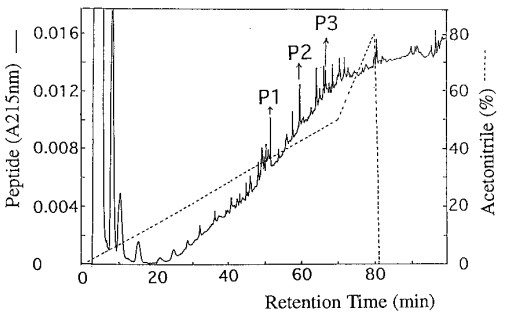

FIG. 3. C_8 RP-HPLC of lysylendopeptidase digests of purified OCIF. Purified homodimer-form OCIF, peak-7 from reverse phase-HPLC (Fig. 1D), was pyridylethylated under reducing conditions and digested with lysylendopeptidase. The digested OCIF was applied to a reverse-phase C_8 column and polypeptides were eluted with a linear gradient of acetonitrile containing 0.1 % TFA. The peptides denoted P1, P2, and P3 were subjected to sequence analysis using a PROCISE Protein Sequencer 494 or 492 (Perkin-Elmer).

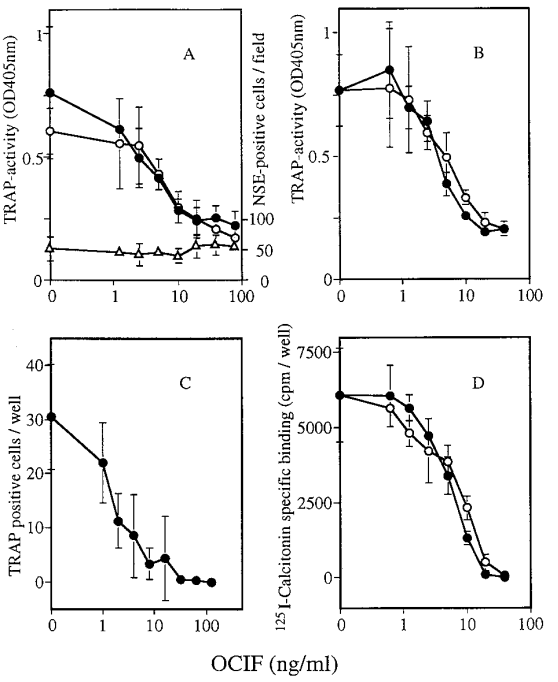

FIG. 4. Effect of OCIF on osteoclast-like cell formation. (A) Effect of OCIF on osteoclast-like cell formation from unfractionated mouse bone cells induced by 1α,25(OH)2D3 or PTH. After incubation for 7 days, TRAP activity in the cells was measured and the number of nonspecific esterase (NSE)-positive cells, the cells stained with an esterase activity assay kit (No. 91-A, Sigma), was counted. The results are expressed as the mean±SD of 15 random microscopic fields ($\times$100 total magnification). Open circle, monomer-form OCIF (peak-6, Fig. 1D) concomitant with 1α,25$(OH)_2D_3$ (TRAP activity) ; Closed circle, monomer-form OCIF with PTH (TRAP activity) ; Open triangle, monomer-form OCIF with 1α,25$(OH)_2D_3$ (nonspecific esterase-positive macrophages). (B) Effect of OCIF on osteoclast-like cell formation induced by 1α,25$(OH)_2D_3$ in cocultures of ST2 stromal cells and mouse spleen cells. After 7 days of incubation, TRAP activity in the cells was measured. Open circle, monomer-form OCIF ; Closed circle, homodimer-form OCIF (peak-7). (C) Effect of OCIF on osteoclast-like cell formation induced by IL-11 in cocultures of PA6 cells and mouse spleen cells. Monomer-form OCIF was added to the cocultures concurrently with 10 ng/ml of IL-11. After 7 days of incubation, the number of TRAP positive osteoclast-like cells was counted. (D) Effect of OCIF on the expression of calcitonin (CT) receptor induced by 1α,25$(OH)_2D_3$ in cocultures of ST2 stromal cells and mouse spleen cells. Expression of CT receptor was determined based on the binding of [^{125}I]-CT as described under "Materials and Methods". Open circle, monomer-form OCIF ; Closed circle, homodimer-form OCIF. All experiments (A, B, C, and D) were performed in triplicate.

slices in the presence of 1α,25$(OH)_2D_3$ (data not shown).

DISCUSSION

OCIF, a novel cytokine which inhibits osteoclast-like cell formation, has been purified to homogeneity from the conditioned medium of human embryonic lung fibroblasts, IMR-90. Addition of CHAPS to the buffers used in the purification steps was crucial in dissocia-

tion of OCIF from other proteins. OCIF is a heparin-binding basic glycoprotein and has been isolated as a monomer with a Mr of 60,000 and a homodimer with a Mr of 120,000.

Three kinds of cytokine, CT (6), IL-4 (20), and interferon-γ (IFN-γ) (20, 21), have been reported to inhibit osteoclastogenesis. The number of CT receptor expressed on osteoclasts increases coinciding with their differentiation stages, and CT binds to its receptor on TRAP positive osteoclasts inhibiting their maturation and bone-resorbing activity (6, 22). OCIF inhibited osteoclast development from immature osteoclast progenitors, while it failed to inhibit bone resorption by mature osteoclasts on ivory slices in the presence of $1\alpha,25(OH)_2D_3$. Recent study demonstrated that IL-4 and IFN-γ act directly on monocytic cells and differentiate them into immune cells via pathway different from osteoclastogenesis (20). Osteoclasts and monocytes/macrophages derive from common bone marrow precursors. Accordingly, the inhibition of osteoclastogenesis by IL-4 and IFN-γ is due to the predominant differentiation of bone marrow precursors into mature monocytes/macrophages. Although local concentration of M-CSF mediates osteoclastic differentiation, addition of exogenous M-CSF to cocultures of ST2 stromal cells and mouse bone marrow cells dose-dependently reduces the number of TRAP positive multinucleated cells and causes maximal 98 % inhibition. This inhibition of osteoclastic differentiation is accompanied by a 2.5-fold increase in the number of nonspecific esterase-positive macrophages (23). In contrast, OCIF strongly inhibited development of osteoclast from unfractionated mouse bone cells induced by $1\alpha,25(OH)_2D_3$ without any increase in the number of nonspecific esterase-positive macrophages (Fig. 4A). In addition, OCIF showed no effect on the development of macrophages induced by exogenous M-CSF (data not shown). The results indicate that the inhibition by OCIF is specific to osteoclast development.

A number of local factors and systemic hormones are involved in osteoclastogenesis through distinct signaling pathways: $1\alpha,25(OH)_2D_3$ through vitamin D receptor; PTH, prostaglandin E2, and IL-1 through activation of protein kinase A; and IL-6, IL-11, oncostatin M, and leukemia inhibitory factor through gp130 (7). It is now suggested that all these stimulators of osteoclastogenesis act on osteoblastic cells to induce an osteoclast differentiation factor (ODF), a hypothetic membrane-bound protein expressed on osteoblastic cells, which recognizes osteoclast progenitors and prepares them to differentiate into mature osteoclasts (6, 7). OCIF dose-dependently inhibited osteoclastogenesis through these three signal transduction pathways in a dose range of 1 to 40 ng/ml and gave almost the same half inhibition concentration (IC50 = 4 to 6 ng/ml). A preliminary binding study using [^{125}I]-OCIF showed that OCIF specifically binds to ST2 stromal cells cultured in the presence of $1\alpha,25(OH)_2D_3$ and dexamethasone (manuscript in preparation). These results suggest such a possibility that OCIF binds to ST2 cells via a membrane-anchored protein involved in the signal transmission between ST2 cells and osteoclastic progenitor cells, eventually suppressing differentiation of progenitor cells into osteoclasts. ODF could be a target of OCIF. Alternatively, OCIF itself may be a ligand transmitting a signal that suppresses production of ODF. Thus, cloning of complementary DNA (cDNA) encoding OCIF, determination of other cells expressing OCIF gene, and the investigation into the OCIF binding molecule on osteoblastic cells are urgent for clarification of the mechanism by which OCIF inhibits osteoclastogenesis.

An attempt to find other activities which OCIF might exert ended unsuccessful. In a dose range of 0.1 to 100 ng/ml, OCIF showed no effect on proliferation of osteoblastic cells (MC3T3-E1 and ST2), other mesenchymal cells (mouse premyeloblastic cells, NFS-60; human monocytic cells, HL-60; human umbilical vascular endothelial cells, HUVEC; and human aorta smooth muscle cells, AOSMC), and epithelial cells (American opossum kidney epithelial cells, OK). In addition, OCIF showed no effect on differentiation of osteoblastic cells (MC3T3-E1), blastogenesis of mouse spleen cells induced by concanavalin A or lipopolysaccharide, and myeloid colony formation from mouse bone marrow cells by murine IL-3 and human erythropoietin. Many of the stromal cell-derived cytokines involved in regulation of osteoclastogenesis are multifunctional. However, OCIF exerted no biological activities besides the osteoclastogenesis inhibitory activity as tested in this study, indicating that OCIF is a cytokine involved specifically in regulation of osteoclastogenesis.

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