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Identification of the Functional Domain of Osteoclast Inhibitory Peptide-1/hSca

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ABSTRACT

Osteoclast (OCL) activity is controlled by local factors produced in the bone microenvironment. We previously identified a novel inhibitor of OCL formation that is produced by OCLs (osteoclast inhibitory peptide-1/human Sca [OIP-1/hSca]). OIP-1/hSca is a glycosylphosphatidylinositol (GPI)-linked membrane protein (16 kDa) that is cleaved from the OCL surface. Immunocytochemical staining further confirmed the expression of OIP-1/hSca in OCL formed in mouse bone marrow cultures. However, the structure/function mechanisms responsible for the inhibitory effects of OIP-1/hSca on OCL formation are unknown. Therefore, we expressed deletion mutants of OIP-1 in 293 cells and tested their effects on OCL formation. These studies indicated that the carboxy-terminal peptide (c-peptide) region is critical for OIP-1/hSca activity. A 33 amino acid OIP-1 c-peptide (10–100 ng/ml) significantly inhibited 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] induced OCL formation and pit formation capacity of OCL on dentine slices in human bone marrow cultures. Furthermore, the c-peptide (10–100 ng/ml) significantly inhibited early human OCL precursor (granulocyte-macrophage colony-forming unit [GM-CFU]) colony formation in methylcellulose cultures. The polyclonal antibody against the OIP-1 c-peptide neutralized the inhibitory effect of OIP-1 c-peptide on OCL formation in mouse bone marrow cultures in vitro. These results show that the OIP-1 c-peptide is the functional domain of OIP-1 and that availability of neutralizing antibody specific to the OIP-1 c-peptide should provide important mechanistic insights into OIP-1/hSca inhibition of osteoclastogenesis in the bone microenvironment. (J Bone Miner Res 2002;17:111–118)

Key words: osteoclast inhibitory peptide-1/human Sca, osteoclast, c-peptide, inhibitors, granulocyte-macrophage colony-forming unit

INTRODUCTION

OSTEOCLASTS (OCLs) are primary bone-resorbing cells that produce factors that stimulate or inhibit OCL formation and activity.[1,2] By screening an OCL-like cell complementary DNA (cDNA) expression library, we recently have cloned and identified OC inhibitory peptide 1/human stem cell antigen (OIP-1/hSca) as a novel inhibitor of OCL formation and bone resorption.[3] OIP-1/hSca also is termed retinoic acid–induced gene expression (RIG-E) or human thymic shared antigen-1 (TSA-1)/Sca-2 and is an Ly-6–related differentiation antigen expressed on immature thymocytes and thymic epithelial cells.[4,5] OIP-1/hSca is a glycophosphatidylinositol (GPI)-linked membrane protein (16 kDa) containing a 79 amino acid extracellular peptide and a 32 amino acid carboxy-terminal GPI-linked peptide (c-peptide). Previously, we have shown that OIP-1/hSca messenger RNA (mRNA) is highly expressed in osteoblastic cells, OCLs, liver, and bone marrow cells. High levels of Sca-2 expression in murine bone marrow cells and spleen also were reported.[6] Recently, it also was shown that Sca-2 is expressed in human lymphoid tissues as well as various nonlymphoid tissues.[7]

We also have shown that OIP-1/hSca is cleavable from the OCL surface and inhibits the OCL formation.[3] It is

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well known that the GPI-anchored proteins are released easily from the cell surface by phosphatidylinositol-specific phospholipase C purified from bacteria. In mammals, the phospholipase D cleaves the inositol phosphate linkage of GPI-anchored proteins.[8] Sca-2 is a useful marker in early T cell development and T cell activation and seems to play a regulatory role in thymocyte differentiation.[9] However, the functions of Sca-2 remain largely obscure. Previously, it has been shown that Sca-2 functions as a modulator of a T cell receptor signaling pathway.[10] An anti–Sca-2 monoclonal antibody (MAb) inhibited tyrosine phosphorylation of CD3 ζ-chains and interleukin-2 (IL-2) production induced by anti-CD3 stimulation in T cell hybridomas,[11] suggesting that a signal via Sca-2 regulates early and late events in T cell receptor signaling. Furthermore, in vivo injection of Sca-2 MAb completely blocked anti–CD3-mediated apoptosis of thymocytes.[12] However, because Sca-2 is a GPI-anchored membrane protein and thus does not have transmembrane and cytoplasmic regions, it is not known how Sca-2 transmits signals into the cytoplasm of the cell. Recently, it has been reported that Sca-2 is associated physically and functionally with CD3 ζ-chains of the T-cell receptor (TCR) complex.[13]

However, the molecular mechanisms responsible for the inhibitory effects of OIP-1/hSca on OCL formation are unknown. Therefore, in this study we determined structure/function characteristics of OIP-1/hSca that are critical for its OCL inhibitory activity.

MATERIALS AND METHODS

DNA transfection kits were obtained from Stratagene (LaJolla, CA, USA). Polymerase chain reaction (PCR) kits were obtained from Perkin Elmer Cetus Corp. (Foster City, CA, USA). All restriction enzymes used were from New England Biolabs, Inc. (Beverly, MA, USA) and chemicals were from Sigma Chemical Co. (St. Louis, MO, USA). Human embryonic kidney epithelial cell line (293 cells) was obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). The 23c6 antibody is a generous gift from Dr. Michael Horton (UK).

OIP-1/hSca cDNA deletion mutant constructs and expression in 293 cells

OIP-1 complete coding sequence and c-peptide region deletion mutant cDNA were PCR amplified using an RIG5CDS sense primer 5′-ACCATGAAGATCTTCTTGCCAGTG-3′, an RIG3CDS antisense primer 5′-GGGGCCAAACCGCAGCAGGGCCGG-3′, and RIG(-GPI) antisense primer 5′-ATTGCACAGAAAGCTCTGGCAGCA-3′, respectively. The PCR reaction mixture of 100 μl containing 2 mmol/liter of $MgCl_2$, 50 mmol/liter of KCl, 10 mmol/liter of Tris-HCl (pH 8.3), 0.2 mmol/liter of each deoxynucleoside triphosphate (dNTP), 2.0 U of Ampli *Taq* DNA polymerase, and 0.1 μmol/liter of sense and antisense primers. The PCR reaction was carried out by incubating the samples at 94°C for 1 minute followed by 35 cycles of 94°C for 1 minute and 60°C for 1 minute, with a final extension for 5 minutes at 60°C. The PCR-amplified Sca-2 cDNA products were subcloned into pcDNA3.1/V5/His-TOPO vector in frame with the V5 epitope[14] tag following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The resulting plasmid constructs OIP-1CDS5–3 native and OIP-1(-GPI) 5–3 deletion mutant were expressed transiently in 293 cells by calcium phosphate method (Stratagene). Serum-free conditioned media collected from these cells were used to assay for its capacity to inhibit OCL formation in murine marrow cultures in vitro as described previously.[16]

Western blot analysis

Two hundred ninety-three cells transiently transfected with OIP-1CDS5–3 native and OIP-1(-GPI) 5–3 deletion mutant plasmid constructs as described previously were lysed in a buffer containing 20 mM of Tris, pH 7.4, 150 mM of NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM of MgCl2, 1mM of EGTA, 200 μM of sodium vanadate, 1 mM of phenylmethylsulfonyl fluoride (PMSF), and 1 mg/ml of aprotinin. Then, samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), using 15% gels (BioRad, Hercules, CA, USA). For immunoblot analysis, proteins were transferred from SDS gels onto a NitroBind nitrocellulose membrane (Micron Separations, Inc., Westboro, MA, USA). After blocking with 5% nonfat dry milk in 150 mM of NaCl, 50 mM of Tris, pH 7.2, and 0.05% Tween 20 (TBST) buffer, the membrane was incubated for 1 h with the mouse MAb (immunoglobulin G2a [IgG2a]) against V5 epitope diluted 1:5000 in 5% nonfat dry milk–TBST. Then, the blots were incubated for 1 h with horseradish peroxidase–conjugated rabbit anti-mouse IgG (Sigma Chemical Co.) diluted 1:10,000 in 5% nonfat dry milk–TBST and developed using an enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, USA).

OCL precursor culture

Nonadherent marrow mononuclear cells were cultured in methyl cellulose to form GM-CFU as described previously.[15] Recombinant human GM-CSF was used as a source of colony-stimulating factor in cultures at a final concentration of 100 pg/ml. The cells (4×10^5/ml) were plated in triplicate and incubated for 8 days in the presence or absence of c-peptide (0–100 ng/ml) or vehicle at 37°C in a humidified atmosphere of 5% carbon dioxide air. Cultures were scored for colonies (aggregates $>$ 50 cells) using an Olympus dissecting microscope (Olympus Optical Co., Tokyo, Japan) at 40$\times$ magnification. The results are reported as the mean $\pm$ SD for triplicate cultures.

OCL-like cell formation

Bone marrow from C57 black mouse tibias that had been removed aseptically was obtained by flushing the tibias with 1 ml of α-minimum essential medium (α-MEM) using a tuberculin syringe fitted with a 27.5G needle. The bone marrow-derived cells were washed twice and resuspended in α-MEM–10% fetal calf serum, and the cells were depleted of cells adherent to plastic by incubating the marrow

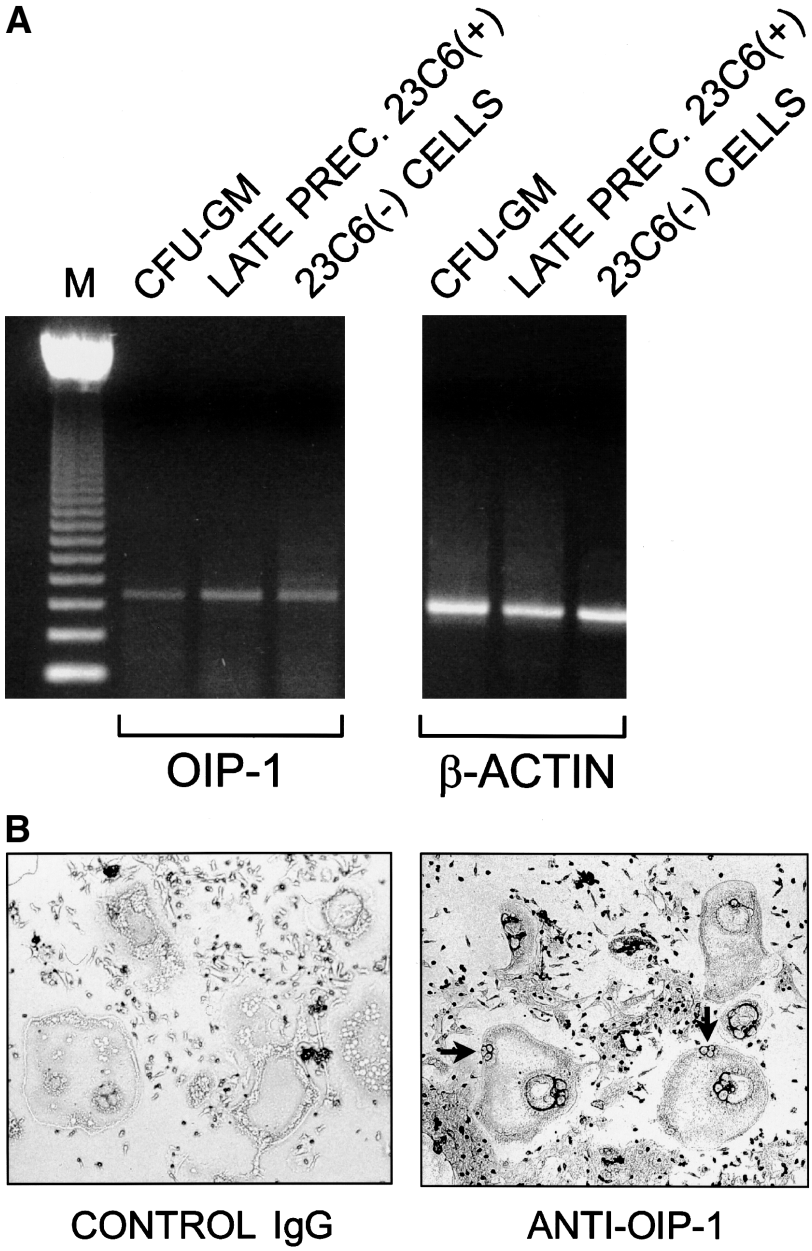

FIG. 1. (A) RT-PCR analysis of OIP-1/hSca mRNA expression in OCL lineage cell types. Total RNA was isolated from human bone marrow–derived OCL early precursors (GM-CFU), late precursors that are anti-vitronectin 23c6 antibody positive, and non-OCL cell types that are 23c6(−) in the left panel. Right panel shows the β-actin amplification in all the cell types analyzed as positive control for RT-PCR analysis. (B) Immunocytochemical staining for OIP-1/hSca expression in OCL-like cells formed in mouse bone marrow cultures. Bone marrow cells were cultured in the presence of recombinant human RANKL (rhRANKL; 50 ng/ml) and mouse macrophage colony-stimulating factor (M-CSF; 25 ng/ml). At the end of a 6-day culture period, the MNCs formed were fixed in 4% formaldehyde and immunostained for OIP-1/hSca expression using rat anti-mouse TSA/Sca-2 antibody (PharMingen) at a concentration of 1 μg/ml as described in the Materials and Methods section. Photomicrographs were taken at magnification ×200. Multinucleated OCL-like cells that were positively stained with anti-Sca-2 antibody were pointed by arrows. No immunostaining was detected with a control rat IgG_1 as shown in the left panel.

cell suspension in sterile 10-cm tissue culture dishes for 2 h. The nonadherent marrow cells were collected and cultured in quadruplicate for 8 days in 48-well plates at a density of 5×10^5 cells/well in α-MEM–10% fetal calf serum supplemented with 10^{-9} M of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] as described by Takahashi et al.[16] The cultures were fixed with 4.5 mM of citric acid, 2.25 mM of sodium citrate, 3 mM of sodium chloride, 3% formaldehyde, and acetone and washed twice in distilled water. Then, the cultures were stained for tartrate-resistant acid phosphatase (TRAP) using an acid phosphatase staining kit (Sigma Chemical). The $TRAP^+$ multinucleated cell (MNC) containing three or more nuclei was counted with an inverted microscope.

Human bone marrow–derived mononuclear cells (10^6/ml) were cultured in a 96-well plate for 3 weeks in the presence of 1,25$(OH)_2D_3$ (10^{-9} M) to form OCL-like cells. At the end of the culture period, anti-vitronectin receptor antibody 23c6 positive OCL-like MNCs were scored as described.[17] Pit formation capacity of OCL on sterile dentin slices in human bone marrow cultures was determined as described previously.[15]

Immunostaining of OIP-1 expression in OCL-like cells

Mouse marrow cells were cultured to form OCL-like MNCs as described previously.[16] At the end of culture period, the cells were fixed in 4% formaldehyde for 30

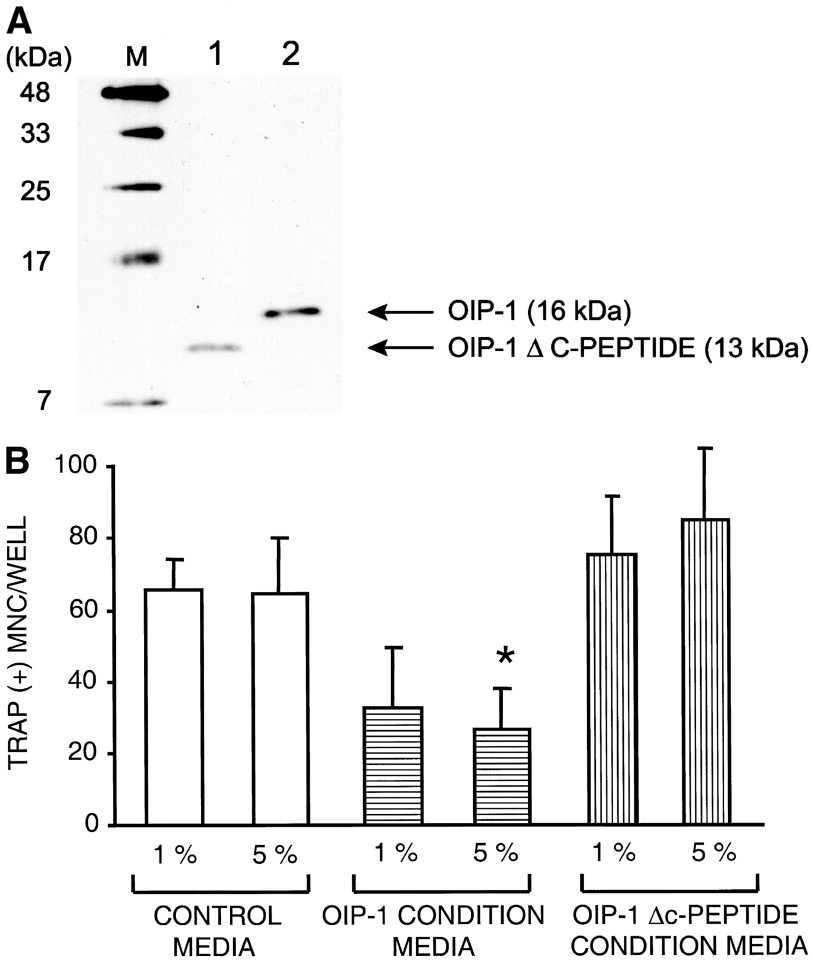

FIG. 2. (A) Western blot analysis of OIP-1/hSca c-peptide deletion mutant protein expression in 293 cells conditioned media. Two hundred ninety-three cells were transiently transfected with the (1) Δ c-peptide deletion mutant and (2) full length OIP-1/hSca expression constructs. After 48 h, the serum-free conditioned media concentrates were subjected to SDS-PAGE (15%) and blot-transferred onto a nitrocellulose membrane. OIP-1/hSca protein was detected by immunoprobing the blot with mouse MAb against V5 epitope as described in the Materials and Methods section (M, :Molecular weight marker). (B) Inhibitory effect of OIP-1/hSca and Δ c-peptide mutant protein on OCL formation in murine bone marrow cultures. Conditioned media obtained from 293 cells transiently transfected with OIP-1/hSca and Δ c-peptide mutant constructs were assayed for OCL inhibitory activity in mouse bone marrow cultures in vitro. Conditioned media obtained from 293 cells that were mock-transfected with empty vector were served as control for these experiments. Mouse bone marrow cells were cultured with $1,25(OH)_2D_3$ (10^{-9} M) in the presence of conditioned media (1% and 5%) samples as described. $TRAP^+$ MNC formed at the end of the culture period were scored (*$p < 0.05$).

minutes. Cells were permeabilized by adding cold phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.1% Triton X-100 for 15 minutes. After preincubation in 1% BSA, rat anti-mouse Sca MAb (PharMingen, San Diego, CA, USA) was added (diluted at 1 μg/ml in PBS and 1% BSA) and continued incubation for 1 h at room temperature. The cells were washed and then incubated with biotinylated rabbit anti-mouse IgG (1:200) for 1 h and stained for OIP-1 expression using an ABC kit from Vector Laboratories (Burlingame, CA, USA) following the manufacturer's protocol.

Neutralization of OIP-1/hSca c-peptide activity

OIP-1/Sca c-peptide (NFSAADGGLRASVTLLGAGLLLSLLPALLRFGP) was synthesized by Genemed Synthesis, Inc. (San Francisco, CA, USA). Polyclonal antibody was raised in rabbits against cysteine keyhole limpet hemocyanin (KLH)-conjugated immunogenic region of OIP-1/hSca c-peptide [(C)NFSAADGGLRASVT] (Zymed Laboratories, Inc., San Francisco, CA, USA). OIP-1 c-peptide–specific polyclonal antiserum was added to mouse bone marrow culture at 1:200 dilution concentration in the presence of 10 ng/ml of concentration of OIP-1 c-peptide and 10^{-9} M $1,25(OH)_2D_3$ as described previously.

RESULTS

OIP-1/hSca gene expression in OCL lineage

Previously, we identified the expression of OIP-1/hSca in OCL-like cells formed in long-term human bone marrow cultures by in situ hybridization.[3] We now further analyzed the expression of OIP-1 in OCL cell lineage types using RT-PCR analysis and immunocytochemistry. As shown in Fig. 1A, RT-PCR analysis of total RNA isolated from early precursors (GM-CFU) and committed OCL precursors (23c6 anti-human vitronectin antibody-positive cells) showed expression of OIP-1/hSca mRNA. Furthermore, RT-PCR analysis also indicated OIP-1 mRNA expression in non-OCL lineage cell types (23c6 antibody-negative cells) present in the human bone marrow cultures. β-actin mRNA was amplified in all the samples analyzed as an internal control for the RT-PCR. Furthermore, immunocytochemical staining using rat MAb against mSca showed positive staining of OCL-like cells and mononuclear cells present in mouse bone marrow cultures (Fig. 1B).

Mapping of OIP-1/hSca functional domain

Native and c-peptide deletion mutant forms of OIP-1/hSca were expressed transiently in 293 cells, and condi-

tioned media was collected after 48 h. The expression of OIP-1/hSca protein in 293 cell lysates was confirmed by Western blot analysis as shown in Fig. 2A. The effect of hSca conditioned media was assayed on murine bone marrow cultures to test their capacity to inhibit OCL-like cell formation in vitro. As shown in Fig. 2B, conditioned media obtained from 293 cells expressing native OIP-1/hSca significantly inhibited OCL-like cell formation. However, conditioned media obtained from 293 cells expressing the c-peptide deletion mutant form of OIP-1 did not inhibit OCL-like cell formation compared with mock transfected cells. These data suggest that OIP-1 c-peptide is critical for the OCL inhibitory activity of OIP-1.

Molecular properties of OIP-1/hSca c-peptide

A 33 amino acid peptide corresponding to OIP-1/hSca-c-peptide was synthesized chemically. Peptide analysis using the ProtParam database search indicates OIP-1 c-peptide showed molecular weight of 3.2 kDa and isoelectric point (pI) 9.6. The peptide contains 30% leucine and 15% alanine residues. The estimated in vivo half-life of the c-peptide following the N-end (Asn residue) rule is 1.4 h.[18] Kyte-Doolittle[19] analysis of c-peptide shows hydrophobicity and poorly immunogenic nature of the peptide. Therefore, synthetic c-peptide was solubilized in isopropanol (1 mg/50 μl) and reconstituted in sterile PBS for working concentrations.

Effect of synthetic OIP-1/hSca c-peptide on OCL precursors and OCL development

Human bone marrow mononuclear cells were cultured in methyl cellulose with GM-CSF in the presence of OIP-1 c-peptide at 0–100 ng/ml concentration as described in the Materials and Methods section. OIP-1 c-peptide significantly inhibits GM-CFU colony formation at 10–100 ng/ml concentration (Fig. 3A). The effect of synthetic c-peptide also was examined on OCL-like cell formation in human bone marrow cultures. OIP-1 c-peptide at a concentration of 10 ng/ml significantly inhibited $1,25(OH)_2D_3$ (10^{-9} M)-stimulated OCL-like cell formation in human bone marrow cultures (Fig. 3B). We also tested the effect of OIP-1 c-peptide on bone resorption capacity of the OCL formed in human bone marrow cultures. Human early OCL precursors (GM-CFU) were cultured on dentine slices to form OCL in the presence or absence of OIP-1 c-peptide. As shown in Fig. 4A, OIP-1 c-peptide at a concentration of 10 ng/ml significantly decreased the number and size of resorption lacunae formed on dentine slices. Furthermore, quantification of these data using a light microscope indicated that there was a 2-fold decrease in number of resorption lacunae formed on dentine slices derived from cultures in the presence of c-peptide compared with control cultures in the absence of c-peptide (Fig. 4B).

Neutralization of OIP-1/hSca c-peptide activity to inhibit OCL formation

We examined the neutralization capacity of OIP-1 c-peptide–specific antiserum to block c-peptide inhibition of OCL formation in mouse bone marrow cultures. When

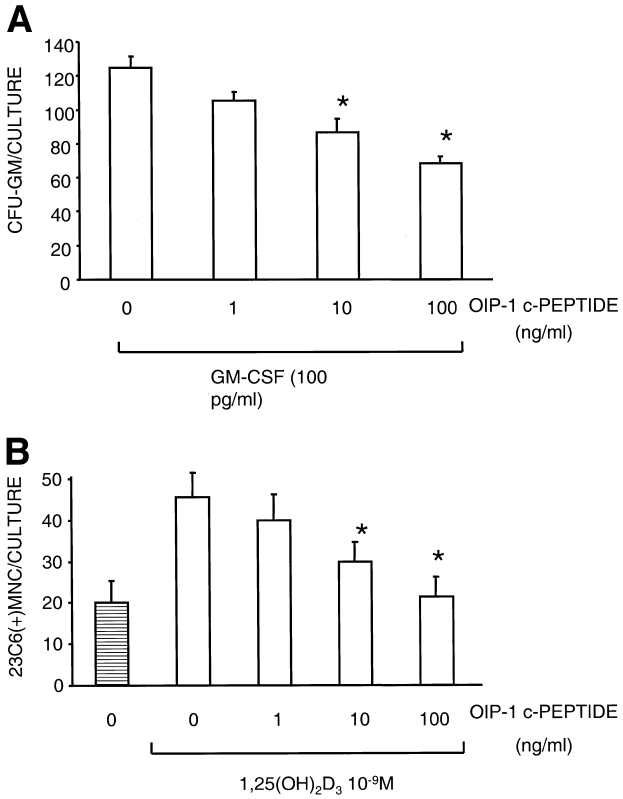

FIG. 3. (A) Inhibitory effect of OIP-1/hSca on OCL precursor GM-CFU colony formation. Human bone marrow cells cultured with hGM-CSF (100 pg/ml) in methyl cellulose (0.7%) to form GM-CFU colonies in the presence or absence of OIP-1 c-peptide. At the end of a 1-week culture period, GM-CFU colonies formed in these cultures were scored using a light microscope (*$p < 0.05$). (B) Effect of OIP-1/hSca c-peptide on OCL-like MNC formation in long-term human bone marrow cultures. Synthetic OIP-1 c-peptide was added to human bone marrow cultures at different concentrations (1–100 ng/ml) in the presence of 10^{-9} M of $1,25(OH)_2D_3$. Vehicle used to solubilize the c-peptide alone served as control. At the end of a 3-week culture period, anti-human vitronectin receptor antibody 23c6-positive MNC formed in these cultures were scored (*$p < 0.05$).

rabbit polyclonal antibody (1:200 dilution) raised against OIP-1 c-peptide was added to murine bone marrow cultures in the presence of OIP-1 c-peptide (10 ng/ml), OCL inhibitory activity of c-peptide was neutralized completely. Interestingly, anti–OIP-1 c-peptide antiserum significantly increased $TRAP^+$ MNC formation in control cultures in the absence of OIP-1 c-peptide (Fig. 5). In contrast, a nonspecific control IgG at 1:100 dilution did not significantly affect $TRAP^+$ MNC formation in these cultures. This further confirms the specificity of OIP-1 c-peptide antiserum to neutralize the OCL inhibitory activity of c-peptide. Therefore, these data suggest that c-peptide is critical for the OCL inhibitory activity by OIP-1.

DISCUSSION

Ly-6 family molecules are low molecular weight GPI-linked peptides with a distinctive cysteine-rich domain and

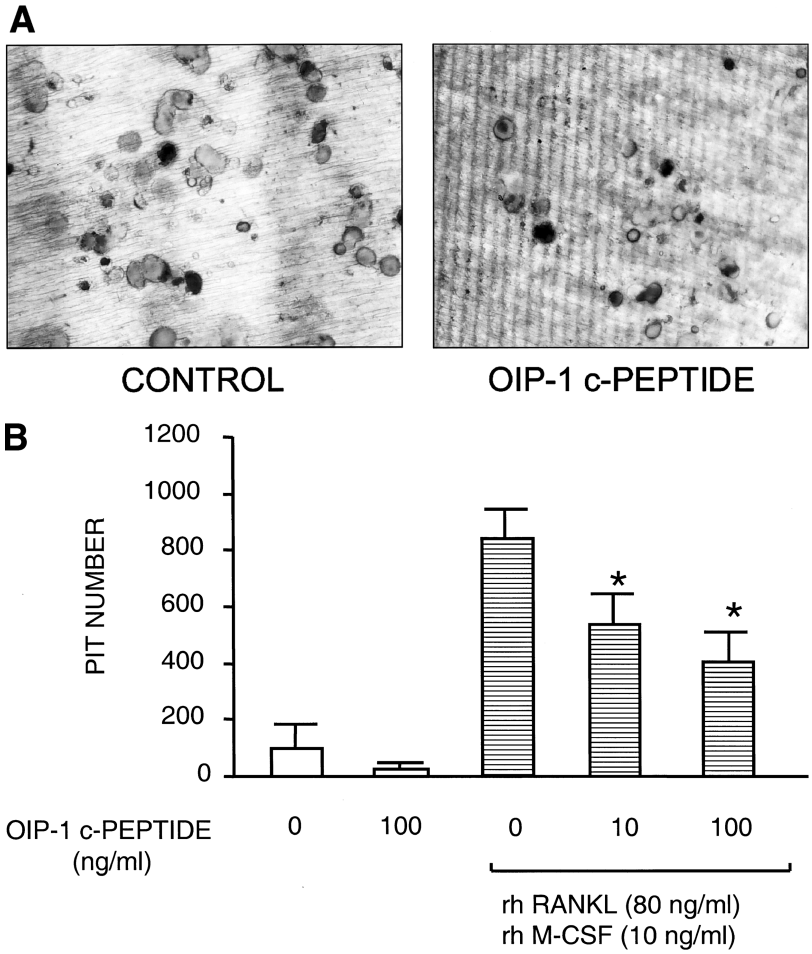

FIG. 4. OIP-1 c-peptide inhibits dentine resorption by OCL formed in human marrow cultures. OCL precursors GM-CFU were cultured over sterile dentine slices with RANKL (80 ng/ml) and macrophage colony-stimulating factor (M-CSF; 10 ng/ml) to form OCL-like MNC as described.[15] (A) Resorption lacunae formed on dentine slices derived from human bone marrow cultures in the presence of OIP-1 c-peptide (10 ng/ml) and control cultures in absence of c-peptide. (B) Quantification of the number of resorption lacunae formed on dentine slices. Results represent the mean ± SEM for quadruplicate determinations for a typical experiment. Similar results were seen in three independent experiments ($*p < 0.05$).

O-linked carbohydrate. Expression of OIP-1/hSca and related Ly6 gene family members in osteoblastic cells[20] suggested a potential role for GPI-linked proteins on bone cell function. We recently reported that OIP-1/hSca mRNA being highly expressed in OCL may function as an autocrine/paracrine inhibitor of OCL formation and bone resorption activity.[3] In this study, immunocytochemical staining of OCL further confirmed the expression of OIP-1 protein in mature OCL. Furthermore, OIP-1 mRNA expression in OCL early and more committed precursors suggest that OIP-1/hSca may play an important role in OCL formation. OIP-1/hSca has no significant homology with the recently described OCL inhibitor, osteoprotegerin, expressed in osteoblastic cells.[21] However, the effects of OIP-1 on receptor activator of NF-κB–receptor activator of NF-κB ligand (RANK-RANKL) signaling mechanism in osteoclastogenesis is yet unclear.[22]

In this study, c-peptide deletion mutant forms of OIP-1 did not inhibit OCL formation, suggesting that the c-peptide is critical for OCL inhibitory activity. The synthetic OIP-1 c-peptide inhibited OCL precursors, GM-CFU growth in methyl cellulose cultures, suggesting that OIP-1 inhibits the proliferative phase of osteoclastogenesis. In addition, the c-peptide inhibited OCL formation by OCL precursors GM-CFU. The capacity of an antibody specific to the OIP-1

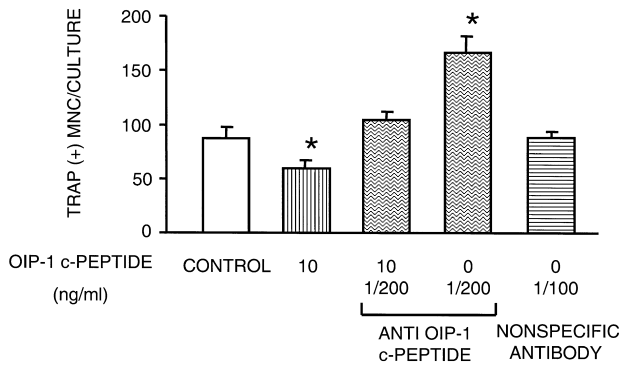

FIG. 5. Neutralization of OIP-1/hSca c-peptide activity to inhibit OCL formation. The ability of rabbit polyclonal antiserum against c-peptide to neutralize the inhibitory effect of OIP-1 c-peptide on OCL formation was assayed in mouse bone marrow cultures. Bone marrow cells were cultured with 10^{-9} M of $1,25(OH)_2D_3$ to form OCL-like cells. c-Peptide antibody was added at 1:200 dilution to these cultures in the presence or absence of OIP-1 c-peptide(10 ng/ml). Nonspecific rabbit IgG at 1:200 dilution was used as control ($*p < 0.05$).

c-peptide to block the effects of OIP-1 further suggests that the inhibitory activity of OIP-1 is mediated by the c-peptide region. The OIP-1 c-peptide antibody significant increase in

OCL formation in the absence of c-peptide suggests that OIP-1 may be present in significant amounts in bone marrow cultures. Furthermore, the OIP-1 c-peptide does not contain an RGD motif, suggesting that the inhibitory activity is not mediated through blocking adhesion molecules such as the vitronectin receptor.[23] Previously, we have shown that OIP-1 does not display toxicity to various types of hematopoietic cells.

The molecular mechanisms responsible for OIP-1/hSca inhibition of OCL formation is unknown. Biochemical and molecular genetic analyses have confirmed that the Ly-6 multigene family is independently regulated and expressed on different hematopoietic cell lineages.[4] The 9804 gene, which encodes a human Ly-6 protein most similar to mouse differentiation antigen TSA-1/Sca-2,[24] has been reported recently. It has been suggested that GPI-anchored proteins transmit signals to the cell interior by interacting with nonreceptor-type tyrosine kinases $p56^{lck}$ and $p59^{fyn}$.[25] GPI-anchored proteins are released easily from the cell surface by phosphatidylinositol-specific phospholipase C– and D–type activities.[8] Bastisch et al.[26] have developed GPI-deficient Jurkat T cells, which are defective in PIG-A gene as a model to study functions of GPI-anchored proteins. This study shows that the OIP-1 c-peptide, which lacks the GPI anchor, has the capacity to inhibit OCL formation, suggesting that the N-terminal portion is not required for OCL inhibition. However, the relative roles of the GPI anchored membrane-bound and -soluble forms of OIP-1/hSca on osteoclastogenesis remain unclear. Ly-6 members have been implicated in cellular activation during hematopoiesis from multipotential stem cells to lineage-committed precursor cells.[27] Furthermore, Ly-6 family members have been shown to be induced transcriptionally by interferons in distinct cell types.[28] Interferons have been shown to inhibit osteoclastogenesis.[29,30] More recently, interferon-γ has been shown to inhibit osteoclastogenesis through rapid degradation of TNF receptor associated factor-6 (TRAF6), which results in strong inhibition of RANKL-induced NF-κB and c-Jun kinase activity.[31] Therefore, further studies will have to determine the regulation of OIP-1/hSca expression and activity by cytokines/growth factors that modulate OCL formation and bone resorption.

In this study, we have detected expression of OIP-1/hSca OCL in non-OCL lineage mononuclear cell types present in bone marrow cultures. TSA-1/Sca-2 has been shown to be expressed on B220, Thy-1, CD4, and Gr-1 antibody-positive cell types present in the bone marrow[6] and virtually all peripheral B cells but is not expressed on peripheral T cells. However, Sca-2 is a unique marker for T cell activation and may affect cytokine production by T cells.[10] It has been postulated that $CD8^+$ T cells may be involved in osteoclastogenesis because depletion of this cell type results in increased OCL formation.[32] Bone marrow and OCL precursors GM-CFU cultures may contain low numbers ($<5\%$) of T lymphocytes. Because T cells have been implicated in OCL formation,[32] it is possible that Sca-2 signaling through TCR may play an important role in T cell–mediated regulation of osteoclastogenesis in the bone microenvironment. IL-18, which has profound effects on T cell activation, has been shown to inhibit OCL formation through increased production of GM-CSF by these cells.[33] However, changes in cytokines/growth factors produced in bone marrow culture that may affect OCL formation in the presence of OIP-1 c-peptide are yet to be elucidated.

In summary, OIP-1/hSca is a potent inhibitor of OCL formation, which is expressed in multinucleated OCLs and precursors. We have identified that the c-peptide region of OIP-1/hSca is critical for inhibition of OCL formation. Furthermore, anti–OIP-1 c-peptide–specific antiserum neutralized completely the OCL inhibitory activity of OIP-1 c-peptide. Therefore, availability of neutralizing antiserum against the functional domain of OIP-1/hSca may provide more insights into the molecular mechanisms associated with OIP-1 to inhibit OCL formation and control of the normal bone-remodeling process.

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