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Osteoclast Inhibitory Peptide-1 (OIP-1) Inhibits Measles Virus Nucleocapsid Protein Stimulated Osteoclast Formation/Activity

Srinivasan Shanmugarajan¹, Rimon F. Youssef¹, Parmita Pati¹, William L. Ries¹, D. Sudhaker Rao², and Sakamuri V. Reddy^{1,*}

¹ Charles P. Darby Children's Research Institute, Medical University of South Carolina, Charleston, South Carolina

² Henry Ford Hospital, Detroit, Michigan

Abstract

Paget's disease (PD) of bone is characterized by increased activity of large abnormal osteoclasts (OCLs) which contain paramyxoviral nuclear and cytoplasmic inclusions. MVNP gene expression has been shown to induce pagetic phenotype in OCLs. We previously characterized the osteoclast inhibitory peptide-1 (OIP-1/hSca) which inhibits OCL formation/bone resorption. OIP-1 is a glycosphosphatidylinositol (GPI)-linked membrane protein containing a 79 amino acid extra cellular peptide and a 32 amino acid carboxy terminal GPI-linked peptide (c-peptide) which is critical for OCL inhibition. In this study, we demonstrate that OIP-1 c-peptide significantly decreased (43%) osteoclast differentiation of peripheral blood mononuclear cells from patients with PD. Also, OIP-1 treatment to normal human bone marrow mononuclear cells transduced with the MVNP inhibited (41%) osteoclast precursor (CFU-GM) growth in methylcellulose cultures. We further tested if OIP-1 overexpression in the OCL lineage in transgenic mice inhibits MVNP stimulated OCL formation. MVNP transduction and RANKL stimulation of OIP-1 mouse bone marrow cells showed a significant decrease (43%) in OCL formation and inhibition (38%) of bone resorption area compared to wild-type mice. Western blot analysis identified that OIP-1 decreased (3.5-fold) MVNP induced TRAF2 expression during OCL differentiation. MVNP or OIP-1 expression did not affect TRAF6 levels. Furthermore, OIP-1 expression resulted in a significant inhibition of MVNP stimulated ASK1, Rac1, c-Fos, p-JNK, and NFATc1 expression during OCL differentiation. These results suggest that OIP-1 inhibits MVNP induced pagetic OCL formation/activity through suppression of RANK signaling. Thus, OIP-1 may have therapeutic utility against excess bone resorption in patients with PD.

Keywords

Paget's disease of bone; osteoclast; measles virus nucleocapsid; OIP-1; RANK ligand

Paget's disease (PD) of bone is characterized by markedly increased osteoclast (OCL) formation and bone resorption followed by excessive new bone formation. Osteoclasts in PD are increased both in number and size, contain paramyxoviral nuclear inclusions, and contain more nuclei per cell than normal OCL. Furthermore, marrow culture studies have identified several abnormalities in OCL formation in PD [Roodman and Windle, 2005]. OCL precursors and circulating peripheral blood cells from Paget's patients express measles virus nucleocapsid

*Correspondence to: Sakamuri V. Reddy, Charles P. Darby Children's Research Institute, 173 Ashley Avenue, Charleston, South Carolina 29425. reddy@muscc.edu.

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(MVNP) transcripts [Reddy et al., 1996]. Also, we previously reported that MVNP gene transduction to normal human OCL precursors results in formation of OCLs with pagetic phenotype [Kurihara et al., 2000]. Further, measles virus (MV) infection of OCL precursors from CD46 transgenic mice form OCL with a pagetic phenotype in vitro [Reddy et al., 2001]. However, viral etiology of PD remains controversial as others have failed to detect paramyxoviral transcripts [Helfrich et al., 2000; Ooi et al., 2000]. Bisphosphonates have offered an advanced therapy for PD of bone. Recent data indicate that a single infusion of zoledronic acid produces high therapeutic response in PD than daily treatment with risedronate [Reid et al., 2005]. Although complications associated with bisphosphonate therapy such as osteonecrosis of the jaw (ONJ) is reported rarely in patients with PD, risk factors and underlying mechanisms of ONJ are still unclear [Bilezikian, 2006].

We have previously identified and characterized a novel inhibitor of OCL formation and bone resorption termed osteoclast inhibitory peptide-1 (OIP-1/hSca) [Koide et al., 2002, 2003]. More recently, we have shown that targeted overexpression of OIP-1 in the OCL lineage develops an osteopetrosis bone phenotype in mice [Shanmugarajan et al., 2007]. OIP-1/hSca is also termed as retinoic acid-induced gene expression (RIG-E) or human thymic shared antigen (TSA-1/Sca-2) is a Ly-6 gene related differentiation antigen expressed on immature thymocytes and thymic epithelial cells [Mao et al., 1996]. OIP-1/hSca is a glycosylphosphatidylinositol (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). We have further identified that the OIP-1 c-peptide region is critical for OCL inhibitory activity [Koide et al., 2002]. It has been suggested that GPI-anchored proteins are membrane bound proteins that can be shed from the cell surface in membrane bound vesicles or cleaved and released by the action of phospholipase C or other proteolytic enzymes. These proteins have been reported to act as cell activators, cell communicators, and signaling molecules in hematopoietic cells [Nosjean et al., 1997]. In addition, GPI linked proteins transmit signals to the cell interior by interacting with non-receptor type tyrosine kinases p56^{lck} and 59^{fyn} [Stefanova et al., 1991]. Moreover, Ly-6A (Sca-1) knock-out mice demonstrated a decreased bone mineral density and bone mineral content [Bonyadi et al., 2003] implicating an essential role for LY-6 gene family in normal bone remodeling. In this study, we determined the mechanism of OIP-1 inhibition of MVNP stimulated pagetic OCL formation and bone resorption activity in vitro.

MATERIALS AND METHODS

MVNP Expression in Bone Marrow Cells

We have previously developed a retroviral plasmid construct, pILXAN#1 that transcribes MVNP mRNA expression under the control of 5' LTR viral promoter elements. The recombinant plasmid construct was transfected into the PT67 amphotropic packaging cell line using the LipofectAMINE (Invitrogen Corp., CA). Stable clonal cell lines producing MVNP recombinant retrovirus at high titer (1×10^6 virus particles/mL) were established by selecting for resistance to neomycin (600 μ g/mL). Similarly, a control retrovirus producer cell line was established by transfecting the cells with the pLXSN empty vector (EV). Producer cell lines were maintained in DMEM containing 10% FCS (Gibco BRL, MD), 100 U/mL each of streptomycin and penicillin, 4 mM L-glutamine, and high glucose (4.5 g/L). Bone marrow cells were transduced with EV or MVNP retroviral supernatants (20%) from the producer cell lines in 4 μ g/mL of polybrene for 24 h at 37°C in a 5% CO₂ incubator as described earlier [Kurihara et al., 2000].

OCL Precursor (CFU-GM) Culture

Normal human ($n = 6$) bone marrow derived non-adherent mononuclear cells were cultured in methylcellulose to form CFU-GM as described previously [Koide et al., 2002]. Briefly, the EV or MVNP transduced non-adherent bone marrow cells ($4 \times 10^5/\text{mL}$) were cultured in Methocult H4230 (Stem cell technologies, Inc., WA) and incubated for 7 days. Cultures were scored for colonies (aggregates >50 cells) using an Olympus dissecting microscope (Olympus Optical Co., Tokyo, Japan) at $100\times$ magnification. The results are reported as the mean $\pm$ SD for triplicate cultures.

Peripheral Blood Mononuclear Cell Culture Assay for OCL Differentiation

Peripheral blood mononuclear cells (PBMC) derived from patients with PD and normal subjects ($n = 6$) were isolated as described [Susa et al., 2004]. All human samples were obtained following the IRB approved protocol at the Medical University of South Carolina. Briefly, 15 mL of whole blood was mixed with 15 mL of warm (37°C) α -MEM, layered over 15 mL of Ficoll-Paque (Sigma, MO) and centrifuged ($1500 \times g$, 30 min) at room temperature. The cell layer on top of the Ficoll-Paque was collected, resuspended in 10 mL of α -MEM and centrifuged. The mononuclear cells collected were plated in 96-well plates at 6×10^5 cells per well in 0.2 mL of medium (α -MEM, pH 7.4, containing 10% FCS) supplemented with 10 ng/mL hM-CSF, 100 ng/mL hRANKL and $1 \mu\text{M}$ dexamethasone. The cells were re-fed twice weekly by semi-depletion (half of the medium withdrawn and replaced with fresh medium). At the end of culture period (17 days) the cells were fixed with 2% glutaraldehyde in PBS and stained for tartrate resistant acid phosphatase (TRAP) activity using a histochemical kit (Sigma). TRAP positive multinucleated cells (MNC) containing three or more nuclei were scored as osteoclast (OCL) cells under a microscope.

OIP-1 Mouse Bone Marrow Culture Assay for OCL Formation/Bone Resorption

We have recently developed transgenic mice targeted with OIP-1 expression in cells of OCL lineage [Shanmugarajan et al., 2007]. Bone marrow cells isolated from wild-type (Wt) and OIP-1 transgenic mice were centrifuged at 1,500 rpm. The cells were resuspended in α -MEM containing 10% FCS and cultured in the presence of 10 ng/mL mM-CSF for 12 h. The non-adherent cells ($1.5 \times 10^6/\text{mL}$) transduced with EV or MVNP were treated with 10 ng/mL mM-CSF for 24 h at 37°C in a 5% CO_2 incubator. The cells were resuspended with fresh α -MEM containing 10% FCS and cultured for 5 days in the presence of 10 ng/mL mM-CSF and 100 ng/mL mRANKL (R&D systems, MN). At the end of the culture period, the cells were fixed with 2% glutaraldehyde in PBS for 20 min and stained for TRAP activity. TRAP positive multinucleated OCLs containing three or more nuclei were scored under a microscope.

To determine the bone resorption activity, the non-adherent bone marrow cells ($1.5 \times 10^6/\text{mL}$) from Wt and OIP-1 mice were transduced with EV or MVNP as described above. The cells were resuspended with fresh α -MEM containing 10% FCS and cultured for 10 days on dentine slices in the presence of 10 ng/mL mM-CSF and 100 ng/mL mRANKL. The cells were removed from the dentine slices using 1 M NaOH, and digital images of the dentine were taken using an Olympus microscope. The areas of resorption lacunae on the digital images were quantified using a computerized image analysis (Adobe Photoshop and Scion MicroImaging version beta 4.2). The percentage of the resorbed area was calculated relative to the total dentine area. All experiments were performed using OIP-1 mice (3–4-week-old) with appropriate litter-mate controls following the IACUC approved procedure at the Medical University of South Carolina, Charleston.

Western Blot Analysis of MVNP Expression and RANK Signaling Molecules

Bone marrow derived non-adherent cells ($1.5 \times 10^6/\text{mL}$) isolated from Wt and OIP-1 mice were transduced with EV or MVNP. The cells were resuspended with fresh α -MEM containing 10% FCS for 48 h in the presence of 10 ng/mL mM-CSF and 100 ng/mL mRANKL. Total cell lysates prepared from the preosteoclast cells were subjected to Western blot analysis for expression of MVNP using rat anti-mouse MVNP antibody (Abcam Inc, MA) and RANK receptor signaling molecules using antibodies against RANK, TRAF2, ASK1, Rac1, c-Fos, NFATc1. β -Actin expression levels in all the samples were used as loading control (Santa Cruz Inc., CA).

Statistical Analysis

Results are presented as mean $\pm$ SD for three independent experiments and were compared by Student's *t*-test. Results were considered significantly different for $P < 0.05$.

RESULTS

OIP-1 Inhibition of OCL Differentiation in Pagetic PBMC Cultures

We have previously characterized OIP-1 inhibition of OCL differentiation and bone resorption in vitro in normal bone marrow cultures and further shown that OIP-1 c-peptide region is critical for OCL inhibitory activity [Koide et al., 2002]. We therefore tested the potential of OIP-1 to inhibit OCL differentiation of PBMC derived from patients with PD. As shown in Figure 1, PBMC derived from patients with PD resulted in significant increase (46%) in TRAP positive MNC compared to normal subjects. OIP-1 c-peptide treatment to pagetic PBMC inhibited 26% and 43% OCL formation at 10 and 100 ng/mL concentrations, respectively. OIP-1 c-peptide treatment thus demonstrated a significant decrease in the enhanced rate of OCL differentiation in pagetic cultures compared to normal PBMC cultures. These results indicate that OIP-1 is an efficient inhibitor of pagetic OCL differentiation in vitro.

OIP-1 Inhibition of MVNP Stimulated CFU-GM Growth

Previously it has been shown that the number of early OCL precursors, CFU-GM, was increased significantly in marrow aspirates from patients with PD compared to normal subjects [Demulder et al., 1993]. Furthermore, it has been demonstrated that MVNP gene expression in normal OCL precursors stimulates OCL formation and induces pagetic phenotype [Kurihara et al., 2006]. Therefore, we next examined if OIP-1 inhibit MVNP stimulation of OCL precursor growth in methylcellulose cultures. Normal human bone marrow derived non-adherent cells were transduced with EV or MVNP gene expression retroviral vectors and cultured in methylcellulose with GM-CSF (10 ng/mL) to form CFU-GM colonies as described in methods. As shown in Figure 2, MVNP transduced marrow mononuclear cells demonstrated a significant increase (38%) in the numbers of CFU-GM colony formation compared to EV transduced cells. OIP-1 treatment to MVNP transduced cells showed a significant decrease (41%) in CFU-GM colony formation. These results indicate OIP-1 suppression of MVNP stimulated OCL precursor growth in vitro.

OIP-1 Inhibition of MVNP Stimulated OCL Differentiation/Bone Resorption

We recently developed mice targeted with OIP-1 expression in cells of the OCL lineage and characterized inhibition of osteoclastogenesis and bone resorption activity in vivo [Shanmugarajan et al., 2007]. Therefore, we further investigated if OIP-1 expression in the OCL lineage inhibits MVNP stimulated OCL formation in bone marrow cultures. The non-adherent bone marrow cells from Wt and OIP-1 mice were transduced with EV or MVNP retroviral vectors and cultured with 10 ng/mL mM-CSF, 100 ng/mL mRANKL for 2 days. Western blot analysis of total cell lysates obtained from the preosteoclast cells showed no

significant difference in the levels of MVNP expression indicating the normalized transduction efficiency (Fig. 3A). MVNP transduction and RANKL stimulation of bone marrow cells derived from OIP-1 mice showed a significant decrease in TRAP positive MNC formation compared to Wt mice (Fig. 3B). Quantification of these results showed that MVNP transduction of Wt mouse bone marrow cells resulted in a 33% increase in OCL formation compared to EV transduced cells. In contrast MVNP enhanced rate of OCL formation is significantly decreased in OIP-1 mouse bone marrow cultures compared to Wt mouse bone marrow cultures (Fig. 3C).

We further examined the bone resorption capacity of OCLs formed in Wt and OIP-1 mice bone marrow cultures transduced with EV or MVNP. MVNP expression in OCL derived from Wt mice showed a significant increase (34%) in resorption area on dentine slices compared to EV transduced cells. In contrast, OCL formed in OIP-1 mouse bone marrow cultures transduced with EV demonstrated a significant decrease (35%) in resorption lacunae on dentine compared to Wt mice. Furthermore, OIP-1 mouse bone marrow cultures transduced with MVNP resulted in a significant inhibition (38%) of OCL bone resorption activity compared with MVNP transduced Wt mouse bone marrow cultures (Fig. 4A and B). These results suggest that OIP-1 inhibit MVNP stimulated OCL formation/bone resorption activity in vitro.

Inhibition of MVNP Stimulated RANK Signaling During OCL Differentiation

RANK-RANKL signal transduction pathway is critical for OCL differentiation, activation, and survival [Takayanagi, 2007]. We previously identified that OIP-1 inhibits OCL formation through RANK receptor signaling mechanism in normal OCL precursor cells [Koide et al., 2003]. We therefore examined the expression levels of RANK receptor signaling molecules such as TRAF2, c-Fos and NFATc1 in response to MVNP expression and RANKL stimulation. Western blot analysis of total cell lysates obtained from the Wt and OIP-1 mice derived preosteoclast cells transduced with EV or MVNP and stimulated with RANKL revealed that OIP-1 expression did not affect the RANK receptor expression in these cells. However, RANK expression was moderately increased in MVNP transduced Wt and OIP-1 mouse derived preosteoclast cells. Further, MVNP stimulated RANK adaptor protein TRAF2 expression (3.5-fold) in preosteoclast cells from Wt mice; however, in contrast TRAF6 expression levels were not affected. Furthermore, MVNP expression did not stimulate TRAF2 expression in OIP-1 mouse derived preosteoclast cells. In addition, c-Fos and NFATc1 transcription factor expression levels were significantly decreased in OIP-1 transgenic mice derived preosteoclast cells (Fig. 5A).

We further examined the c-Jun N-terminal kinase (JNK) activation in response to MVNP stimulation in OIP-1 mouse bone marrow cells. As shown in Figure 5B, OIP-1 mouse derived preosteoclast cells demonstrated suppression of JNK phosphorylation compared to Wt mice. However, MVNP expression did not stimulate JNK phosphorylation in OIP-1 mouse bone marrow cells in response RANKL stimulation. In contrast, MVNP expression in Wt mouse bone marrow derived preosteoclasts showed a 2.5-fold increase in JNK phosphorylation compared to EV transduced cells. In addition, the expression of JNK activators such as Rac1 and ASK1 expression levels were suppressed in OIP-1 preosteoclast cells compared to Wt mice. Further, MVNP expression in Wt preosteoclasts induced 3.6 and 3.2-fold increase in Rac1 and ASK1 expression, respectively. However, there is a significant decrease in MVNP stimulation of Rac1 and ASK1 expression in OIP-1 preosteoclast cells (Fig. 5C). Taken together, these results indicate that OIP-1 inhibits OCL formation and bone resorption activity through suppression of RANK signaling in MVNP stimulated OCL differentiation.

DISCUSSION

Ultrastructural and immunocytochemical studies identified expression of paramyxoviral nucleocapsid antigens in pagetic OCL [Roodman and Windle, 2005]. Bone marrow culture

techniques further identified several abnormalities in OCL and precursors cells from patients with PD. The number of early OCL precursors, CFU-GM, was increased significantly in marrow aspirates from patients with PD compared to normal subjects. Also, it has been shown that OCL formed more rapidly with increased numbers (10–100-fold) of nuclei per OCL in marrow cultures from patients with PD compared to normal subjects [Demulder et al., 1993]. Although recurrent mutations occur in the UBA domain of Sequestosome 1 (SQSTM1/p62) in patients with PD [Laurin et al., 2002; Layfield et al., 2004], recent evidence indicated that mutant p62 expression is not sufficient to induce pagetic phenotype in OCL, but may cause a predisposition to the development of PD through enhanced sensitivity of OCL precursors to osteoclastogenic cytokines [Kurihara et al., 2007]. Lack of skeletal abnormalities in p62 deficient mice [Duran et al., 2004] further suggests a genetic defect may favor the environmental factors such as MV infection to have a potential role in abnormal OCL development in PD. However, the molecular basis for the abnormalities associated with OCLs and the role of paramyxoviral infection in patients with PD is unclear.

In this study, our results indicating that OIP-1 is an inhibitor of MVNP stimulated OCL progenitor growth and differentiation implicate a potential therapeutic utility for high bone turnover associated with PD. Pagetic OCL produce increased levels of IL-6 and express high levels of IL-6 receptors compared to normal subjects. Coculture studies of OCL precursors and cells from the marrow microenvironment of patients with PD and normals have demonstrated that the marrow microenvironment is more osteoclastogenic than normal [Reddy, 2006]. However, we show that OIP-1 transgenic mouse bone marrow cells targeted with OIP-1 expression in cells of the OCL lineage did not respond to MVNP stimulation of OCL formation/bone resorption activity. Therefore, OIP-1 expression in OCL may have a direct effect on signaling pathways and gene expression critical for OCL differentiation. Previously it has been shown that the levels of IL-6, c-Fos, Bcl 2 anti-apoptotic gene mRNA expression in pagetic OCLs are elevated [Brandwood et al., 2003; Roodman and Windle, 2005]. Therefore, gene array profiling studies using OIP-1 mice derived OCL further provide insights into MVNP altered gene expression and pagetic phenotype in OCL.

RANKL is a critical OCL differentiation factor that is expressed on marrow stromal and osteoblast cells in response to several osteotropic factors. It has been shown that marrow stromal cells from pagetic lesions have increased RANKL expression and that OCL precursors from patients with PD are hyper-responsive to RANKL [Menaar et al., 2000; Neale et al., 2002]. Further, enhanced levels of TAF_{II}-17 expression is responsible for the hypersensitivity of pagetic OCL precursors to 1,25-dihydroxyvitamin D₃ [Kurihara et al., 2004]. Gene expression profiling studies indicated that IFN- γ and associated signaling molecules are upregulated in pagetic PBMC and in contrast TNF- α is downregulated [Nagy et al., 2007]. Although IFN- γ is a direct inhibitor of OCL differentiation, recent evidence indicates that IFN- γ stimulates osteoclastogenesis through enhanced levels RANKL levels in T-lymphocytes in vivo [Kotake et al., 2005; Gao et al., 2007]. Therefore, OIP-1 inhibition of RANK receptor signaling in OCL precursor cells counteracts osteoclastogenic factors such as IL-6, RANKL, M-CSF that are elevated in PD [Neale et al., 2002; Reddy, 2006].

The RANK-RANKL signaling promotes the binding of TNF receptor associated factor (TRAF) family proteins such as TRAF-6 to RANK receptor, resulting in activation of NF- κ B and Jun N-terminal kinase (JNK) pathways [Takayanagi, 2007]. Previously, it has been shown that MVNP enhance OCL formation through enhanced levels of NF- κ B and JNK levels [Kurihara et al., 2000]. However, we have demonstrated that the OIP-1 signaling mechanism is independent of NF- κ B activation and involves suppression of p-c-Jun kinase to inhibit OCL formation [Koide et al., 2003]. Also, consistent to our previous results, OIP-1 mice derived preosteoclast cells demonstrated a significant decrease in TRAF-2 expression but had no change in the levels of TRAF-6 (data not shown) and RANK expression in these cells.

Therefore, OIP-1 inhibition of p-c-*Jun* kinase activity is mediated by TRAF-2. Transcription factors such as c-Fos and NFATc1 which modulate target gene expression are critical for OCL development and bone resorption activity [Ikeda et al., 2004]. We show that MVNP expression significantly elevated NFATc1 expression, however, in contrast no significant change in c-Fos expression occurred in OCL precursor cells derived from Wt mice. c-Fos expression levels are moderately decreased in OIP-1 mouse preosteoclast cells compared to Wt mice. Interestingly, MVNP expression did not stimulate NFATc1 expression in OIP-1 mouse derived preosteoclasts indicating that OIP-1 is a potent inhibitor of the RANK receptor signaling pathway critical for OCL differentiation. This is consistent with our recent report that mice targeted with OIP-1 overexpression in cells of OCL lineage develop an osteopetrosis bone phenotype due to inhibition of OCL formation activity in vivo [Shanmugarajan et al., 2007]. It has been reported that ASK1 is activated by TRAF2, TRAF5, and TRAF6 overexpression and mediates TRAF2 induced JNK activation [Nishitoh et al., 1998]. Similarly, Rac has been shown to activate JNK and p38 MAPK pathways [Caron and Hall, 1998]. In the present study, we show that MVNP expression significantly increased Rac1 and ASK1 signaling molecules in Wt mouse derived preosteoclast cells. However, OIP-1 mouse derived preosteoclast cells demonstrated suppression of Rac1, ASK1, and JNK activity further suggesting that OIP-1 inhibits MVNP stimulated OCL formation through suppression of TRAF2 dependent JNK activity. Thus, OIP-1 may have therapeutic utility against excess bone resorption activity in patients with PD.

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Fig. 1.

OIP-1 inhibits osteoclast formation in PBMC cultures from patients with PD. PBMC derived from normal and PD subjects ($n = 6$) were cultured to form OCL in the presence of 10 ng/mL hM-CSF, 1 μ M dexamethasone and 100 ng/mL hRANKL with or without OIP-1 cpeptide at different concentrations (0–100 ng/mL) as described in the methods. The results represent quadruplicate cultures of five independent experiments and data shown as mean $\pm$ SD, ($P < 0.05$).

Fig. 2.

OIP-1 inhibits MVNP stimulated CFU-GM formation in human bone marrow cultures. Non-adherent human ($n = 6$) bone marrow cells ($4 \times 10^5/\text{mL}$) transduced with MVNP or empty vector (EV) were cultured with GM-CSF (10 ng/ml) in the presence or absence of OIP-1 (100 ng/ml) in methylcellulose to form CFU-GM colonies. At the end of a 7-day culture period, CFU-GM colonies formed in these cultures were scored using a light microscope. The results represent quadruplicate cultures of five independent experiments and data shown as mean $\pm$ SD, ($P < 0.05$).

Fig. 3.

Osteoclast formation in wild-type (Wt) and OIP-1 transgenic mouse bone marrow cultures transduced with MVNP or empty vector (EV). **(A)** The non-adherent bone marrow cells from Wt and OIP-1 mice were transduced with EV or MVNP retroviral vectors as described in the methods and cultured with 10 ng/mL mM-CSF, 100 ng/mL mRANKL for 2 days. Total cell lysates were prepared from these preosteoclast cells and MVNP expression was confirmed by Western blot analysis using rabbit anti-MVNP antibody. **(B)** Bone marrow cells from Wt and OIP-1 transgenic mice were transduced with MVNP or EV and cultured for OCL formation in the presence of 10 ng/mL mM-CSF and mRANKL (100 ng/mL) for 4 days. At the end of the culture period the cells were fixed and stained for TRAP activity. **(C)** The TRAP (+) multinucleated cells (MNC) formed were scored under a light microscope. The results represent quadruplicate cultures of three independent experiments and data shown as mean $\pm$ SD, ($P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 4. OIP-1 inhibits MVNP stimulated OCL bone resorption activity. **(A)** Wild-type (Wt) and OIP-1 mouse bone marrow derived non-adherent cells (1×10^6) transduced with MVNP and EV were cultured on dentine slices for 10 days in the presence of 10 ng/mL mM-CSF and 100 ng/mL mRANKL. At the end of the culture period, the cells were removed and stained with toluidine blue. **(B)** The percentage of the mineralized surface area resorbed was quantified as described in the methods. The results represent quadruplicate cultures of three independent experiments and data shown as mean $\pm$ SD, ($P < 0.05$).

Fig. 5. Western blot analysis of RANK receptor signaling molecules in MVNP transduced OIP-1 mice derived preosteoclast cells. Bone marrow cells from Wt and OIP-1 mouse were transduced with MVNP or EV and cultured in the presence of 10 ng/mL mMCSF and 100 ng/mL mRANKL for 2 days and the total cell lysates prepared were subjected to Western blot analysis for RANK receptor signaling molecules.