Published in final edited form as:

Gene. 2006 April 12; 371(1): 16–24. doi:10.1016/j.gene.2005.11.001.

Functional characterization of human osteoclast inhibitory peptide-1 (OIP-1/hSca) gene promoter

Shanmugarajan Srinivasan^a, Masahiro Ito^b, Hiroshi Kajiya^b, L. Lyndon Key Jr.^a, Theresa L. Johnson-Pais^c, and Sakamuri V. Reddy^{a,*}

^aChildren's Research Institute, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425, USA

^bDivision of Hematology-Oncology, University of Pittsburgh, Pittsburgh, PA 15237, USA

^cDepartment of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX 78229, USA

Abstract

We have recently identified and characterized the human osteoclast (OCL) inhibitory peptide-1 (OIP-1/hSca), a member of Ly-6 gene family. OIP-1 is an important physiologic regulator of OCL development and bone resorption activity. To determine the molecular mechanisms that regulate OIP-1 gene expression in OCL precursor cells, we isolated and characterized the OIP-1/hSca gene (2 Kb) promoter sequence. IFN- γ (50 ng/ml) treatment of RAW 264.7 macrophage cells transfected with OIP-1 gene (–1 to –1988 bp relative to transcription start site) promoter-luciferase reporter plasmid demonstrated a significant (4 fold) increase in OIP-1 gene promoter activity. Sequence analysis of OIP-1 gene promoter region further identified a potential Stat-1 binding motif at –1629 to –1639 bp position. Stat-1 specific inhibitor, fludarabine (50 μ M) abolished IFN- γ stimulated OIP-1 gene promoter activity. Electrophoretic mobility shift assay (EMSA) further confirmed activated Stat-1 binding to the OIP-1 gene promoter sequence suggesting that IFN- γ regulates OIP-1 gene expression in OCL precursor cells through a Stat-1 dependent signaling pathway. We further show that knock-down of TRADD enhances IFN- γ signaling to increase OIP-1 gene expression in OCL precursor cells. These results should provide insights into the molecular control of OIP-1 gene expression and inhibition of OCL activity in the bone microenvironment

Keywords

Osteoclast inhibitory peptide-1 (OIP-1/hSca); Interferon gamma; Promoter; Stat-1; Transcription factor

1. Introduction

We have recently identified and characterized a novel inhibitor of osteoclast (OCL) formation termed OCL inhibitory peptide-1 (OIP-1/hSca) (Koide et al., 2002, 2003). OIP-1/hSca is also termed, RIG-E (retinoic acid induced gene expression) or thymic shared antigen-1 (TSA-1) and is a Ly-6 gene family member expressed on immature thymocytes and thymic epithelial cells (Mao et al., 1996). OIP-1 shows 65% homology with mouse Sca-2. We have demonstrated that the OIP-1 c-peptide region is critical for its OCL inhibitory activity and that a neutralizing

antibody against the c-peptide completely blocks OIP-1's capacity to inhibit OCL formation (Koide et al., 2002).

Sca-2, a murine homolog of OIP-1 is a marker gene expressed during early T cell development and T cell activation and seems to play a regulatory role in thymocyte differentiation (MacNeil et al., 1993). However, the functions of Sca-2 remain largely obscure. Previously, it had been demonstrated that Sca-2 functions as a modulator of the T cell receptor signaling pathway (Kosugi et al., 1994). An anti-Sca-2 monoclonal antibody inhibited tyrosine phosphorylation of CD3 ζ chains and IL-2 production induced by anti-CD3 stimulation in T cell hybridomas, suggesting that a signal via Sca-2 regulates early and late events in T cell receptor signaling (Saitoh et al., 1995). Furthermore, in vivo injection of Sca-2 mAb completely blocked anti-CD3 mediated apoptosis of thymocytes (Noda et al., 1996). However, since Sca-2 is a glycosylphosphatidylinositol (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 physically and functionally associated with CD3 ζ chains of the TCR complex (Kosugi et al., 1998). It has also been suggested that GPI-anchored proteins transmit signals to the cell interior by interacting with non-receptor type tyrosine kinases p56^{lck} and p59^{fyn} (Stefanova et al., 1991). Ly-6A (Sca-1) knock-out mice demonstrated that bone mineral density and bone mineral content in Sca-1 null mice were significantly lower than wild type mice at 12 months period (Bonyadi et al., 2003). These studies implicate an essential role for Ly-6 gene family in normal bone remodeling.

Immune cell products such as IFN- γ are potent inhibitors of OCL formation. We have previously shown that IFN- γ stimulates OIP-1/hSca expression in OCL precursor cells (Koide et al., 2003). However, IFN- γ modulates gene expression through complex regulatory mechanisms. Therefore, in the present study, we have isolated the OIP-1 gene promoter sequence and determined the molecular mechanism by which IFN- γ regulate OIP-1 gene expression in OCL precursor cells. These studies should allow development of transgenic mouse models in which transactivating factors for the OIP-1 gene are over expressed in cells of OCL lineage, to determine if they modulate OIP-1 expression and OCL development in the bone microenvironment. Identification and characterization of factors that modulate OIP-1 activity and gene expression are critical to our overall understanding of the regulation of OCL development in both normal and pathologic bone remodeling, and to control excess bone loss associated with several bone diseases including osteoporosis and hypercalcemia of malignancy.

2. Materials and methods

2.1. Isolation and characterization of the OIP-1/hSca gene promoter sequence

We have screened the RPCI-11 human BAC library (BAC-PAC Resources, Childrens Hospital, Oakland, CA) DNA spotted at high density onto hybridization filters by Southern hybridization (Kim et al., 1996). PCR amplified OIP-1 gene upstream sequences using Scap(5) 5' CCCCTCCCTGGTGCTCGCCCTGTC 3' and Scap(3) 5' TCACTGACACCTGGACAGTCCTCG 3' sense and anti-sense primers respectively were ³²P radiolabelled and used as probes. The BAC genomic clone (314A1) encoding OIP-1/hSca was thus isolated. Restriction endonuclease digestion and subsequent hybridization with OIP-1 gene specific probes further identified a 4 Kb Bst $\times$ 1 DNA fragment overlapping the previously reported 1.0 Kb sequence of the 5'-flanking region of the hSca gene (Shan et al., 1998). Therefore, OIP-1 promoter sequence (−1 to −1988 bp relative to transcription start site) was PCR amplified using the sense primer (S4) 5' GTGCTCGAG GCAGTATTGCTGGGAAAGAGGGTC 3' (from 2167 to −2190 bp and Xho I enzyme site added is underlined); anti-sense primer (3)- 5' AGCCTCCCAGGCAGCCGCGCGCCCCGG

3' (from +2 to -25 bp relative to ATG start codon). The DNA fragment was further sub-cloned into the TA vector (plasmid clone OIP-1 TA#8) and the OIP-1/hSca gene 5'-flanking region was sequence analyzed (GenBank Accession# AY857959). The OIP-1 promoter sequence (-1 to -1988 bp relative to transcription start site) was sub-cloned at Sma I restriction enzyme site of pGL2 Basic vector containing the luciferase reporter gene (OIP-1 pGL2 Basic#3).

2.2. Electrophoretic Gel Mobility Shift Assay (EMSA)

The OIP-1 gene promoter sequence containing Stat binding region (-1629 to -1639 bp position), sense 5'-GGTGAGCAAGCGTTCCTCTAACAGTGGGAA-3' and anti sense 5'-TTCCCACTGTTAGAGGAACGCTTGCTCACC-3' were heated up to 94 °C for 2 min and cooled at room temperature for 10 min. The sequence of the duplex oligonucleotide was ³²P radiolabeled and used as probe. To confirm the specificity of EMSA reaction, we have also developed probe in which the Stat-1 binding site was mutated as underlined (Mut-oligo Sense 5' GGTGAGCAAGCGTTAAATTAACAG TGGGAA 3'). Nuclear extracts were prepared from RAW 264.7 macrophage cells and preosteoclast cells stimulated with IFN- γ (50 ng/ml) for 24 h period as described (Koide et al., 2003). The DNA binding reaction was performed in a volume of 15 μ l, which contained the binding buffer (10 mM Tris-HCl at pH 5.5, 0.5 mM EDTA, 1 mM MgCl₂, 4% glycerol, 50 mM NaCl, 0.1 mM Na₃VO₄ and 0.5 mM dithiothreitol), 50 μ g/ml of Poly (dI-dC), 10⁵ cpm of ³²P-labeled probe and nuclear extract (30 μ g). After incubation for 30 min at 4 °C, the samples were analyzed by electrophoresis in a 5% native polyacrylamide gel with 10 mM Tris-glycine (pH 8.6) buffer followed by autoradiography.

2.3. Western blot analysis

Expression of Stat-1 and TRADD in RAW 264.7 cells and mouse bone marrow derived non-adherent cells cultured in the presence of M-CSF (10 ng/ml) for 24h (preosteoclast cells) (Wada et al., 2005) were analyzed by Western blot analysis. Briefly, RAW 264.7 macrophage cells or preosteoclast cells stimulated with IFN- γ (50 ng/ml) for indicated period were lysed in a buffer containing 20 mM Tris-HCl at pH 7.4, 1% Triton X-100, 1 mM EDTA, 1.5 mM MgCl₂, 10% glycerol, 150 mM NaCl, 0.1 mM Na₃VO₄ and 1 $\times$ protease inhibitor cocktail obtained from Sigma. The protein content of the samples was measured using the BCA protein assay kit (Pierce). Protein (20 μ g) samples were then subjected to SDS-PAGE using 12% Tris-HCl gels. The proteins were transferred from SDS gels on to a nitrocellulose membrane. Blocking was performed with 5% BSA in TBS-T (Tris, pH7.2, 150 mM NaCl; 0.1% Tween 20). The membrane was incubated with rabbit polyclonal antibody against Stat-1 and TRADD at 1:1000 dilution (Santa Cruz Biotechnology Inc, Santa Cruz,CA). The blots were incubated for 1h with horseradish peroxidase conjugated goat anti-rabbit antibody diluted 1:2500 in TBS-T buffer, and developed using ECL system (Amersham Biosciences).

2.4. Immunostaining of pStat-1

RAW 264.7 cells were cultured in Lab-Tek Chamber slides (Nunc Inc., Naperville, IL) and treated with or without IFN- γ (50 ng/ml) for 30 min and fixed with 4% paraformaldehyde for 30 min. Cells were permeabilized by adding cold phosphate buffered saline containing 0.5% BSA and 0.1% Triton X-100 for 15 min. Endogenous peroxidase was inactivated by 0.3% H₂O₂ in methanol. Blocking was performed in 1% BSA in saline. The cells were incubated with rabbit anti pStat-1 antibody at 1:1000 dilution for 1 h. The cells were washed and incubated for 1 h with horseradish peroxidase conjugated bovine anti-rabbit antibody (Santa Cruz Biotechnology Inc, Santa Cruz,CA) at 1:2500 dilution. Peroxidase activity was detected by 3'-diaminobenzidine in the presence of 0.1% hydrogen peroxide.

2.5. siRNA interference

To assess the influence of TRADD (Tumor necrosis factor Receptor 1 Associated Death Domain protein) on OIP-1 gene promoter activity, we performed siRNA interference (Hoshika et al., 2005) to suppress TRADD expression in OCL precursor cells. Briefly, RAW264.7 cells were seeded (5×10^5 cells/well) in 6 well plates and supplemented with α -MEM containing 10% fetal calf serum. A day after seeding, cells were cotransfected with double stranded TRADD siRNA (10 μ M) containing 21 nucleotide (Santa Cruz Biotechnology, INC, CA) and OIP-1 pGL2 Basic#3 reporter plasmid DNA (2 μ g) by Lipofectamine method (Invitrogen Life Technologies, Inc., Carlsbad, CA). After 48 h, cells were incubated with serum-free medium with or without IFN- γ (50 ng/ml) for 2 h period. Luciferase activity levels were measured (Promega Inc., Madison, WI) in the total cell lysates prepared and transfection efficiency was normalized by measuring β -galactosidase activity co-expressed in these cells. Suppression of TRADD expression by siRNA was confirmed by Western blot analysis using rabbit anti-TRADD antibody (Santa Cruz biotechnology Inc, CA) as described above.

2.6. Quantitative real-time RT-PCR

Real time PCR was performed as described previously (Khan et al., 2004). Briefly, total RNA was isolated from mouse bone marrow derived preosteoclast cells stimulated with IFN- γ for indicated period (0–48 h) using RNeasy reagent (Qiagen, Crawfordsville, IN) according to the manufacturer's protocol. Reverse transcription reaction was performed using poly-dT primer and Moloney murine leukemia virus reverse transcriptase (Applied Biosystem) in 18 μ l reaction volume containing total RNA (5 μ g), 1 $\times$ PCR buffer and 2 mM $MgCl_2$ at 42 $^{\circ}C$ for 15 min followed by 95 $^{\circ}C$ for 5 min. The quantitative real-time PCR was performed using IQTM SYBR Green Supermix in an iCycler (iCycler iQ Single-color Real Time PCR detection system; BioRad, Hercules, CA). The primer sequences used for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were 5' CCTACCCCAATGTATCCGTTGTG-3' (sense) and 5'-GGAGGAATGGGAGTTGCTGTTGAA-3' (anti-sense) and primers for OIP-1 were 5' TTTATTACCCAGGAGGAGCTGAAGGTTCC3' (sense) and 5' CCAACATGAGAGTCTTCCTGCCTG3' (anti-sense). Thermal cycling parameters were 94 $^{\circ}C$ for 3 min, followed by 40 cycles of amplifications at 94 $^{\circ}C$ for 30 s, 66 $^{\circ}C$ for 1 min, 72 $^{\circ}C$ for 1 min, and 72 $^{\circ}C$ for 5 min as the final elongation step.

3. Results

3.1. Cloning and characterization of OIP-1/hSca gene promoter

To determine the molecular mechanisms underlying the OIP-1/hSca 1 gene expression in OCL precursor cells, we have isolated a BAC genomic clone (314A1) encoding the OIP-1/hSca as described in the methods. Southern blot analysis of the BAC OIP-1 genomic clone using the ^{32}P radio-labelled OIP-1 cDNA probe further confirmed the specificity of the isolated clone encoding the human OIP-1 gene (Fig. 1, *left panel*). We further isolated a 4 Kb Bst $\times$ I DNA fragment overlapping the previously reported 1.0 Kb sequence of the 5'-flanking region of the hSca gene, however no promoter activity been demonstrated (Shan et al., 1998). A partial restriction enzyme digestion map of the OIP-1 promoter region was generated (Fig. 1, *right panel*) and a 2 Kb OIP-1 gene promoter region relative to the transcription start site was sequence analyzed (*GenBank Accession No.* AY857959). Nucleotide sequence analysis using TFSEARCH database search further identified several regulatory motifs with significant identity (>85%) for transcription factors such as SP-1, SRF, c-Myb, GATA, AP-1 and AP-4 in the OIP-1 gene promoter region. We have also identified a potential Stat-1 binding motif at -1629 to -1639 bp position relative to the transcription initiation site in the OIP-1 gene promoter region (Fig. 2).

3.2. IFN- γ regulation of OIP-1 gene promoter activity

We have previously shown that IFN- γ induces OIP-1 mRNA expression in OCL precursor cells (Koide et al., 2003). To further identify the transcriptional regulatory mechanisms operative in OCL precursor cells, we have developed OIP-1 gene promoter-luciferase reporter construct as described in methods. We have previously shown that IFN- γ treatment inhibits RANKL induced OCL differentiation of RAW 264.7 cells (Koide et al., 2003). Therefore, OIP-1 pGL2 Basic#3 luciferase reporter plasmid containing the OIP-1 gene promoter sequence (–1 to –1988 bp relative to transcription start site) was transiently transfected into RAW 264.7 cells and measured the luciferase activity levels in response to IFN- γ treatment. IFN- γ treatment demonstrated a significant increase (4 fold) in OIP-1 gene promoter activity in these cells (Fig. 3A). In contrast, RANKL alone or in combination with IFN- γ treatment to these cells did not significantly affect OIP-1 gene promoter activity (data not shown). Therefore, these results indicate that IFN- γ induces OIP-1 gene promoter activity in RAW 264.7 cells. IFN- γ has been shown to regulate gene expression through Stat-1 dependent and independent signaling (Ramana et al., 2002). We further examined the participation of Stat-1 signaling molecule in IFN- γ stimulated OIP-1 gene expression. Sequence analysis identified the presence of a potential Stat-1 binding motif at –1629 to –1639 bp position relative to the transcription initiation site of the OIP-1 gene promoter. We therefore tested the ability of a Stat-1 specific inhibitor, fludarabine to block IFN- γ stimulation of OIP-1 gene promoter activity. IFN- γ stimulation of RAW264.7 cells transfected with the OIP-1 pGL2 Basic#3 promoter (–1 to –1988 bp)-luciferase reporter construct in the presence of Stat-1 inhibitor, fludarabine (50 μ M) decreased IFN- γ stimulated OIP-1 gene promoter activity (Fig. 3B). However, IFN- γ treatment did not stimulate the activity of an OIP-1 gene promoter deletion construct (–1 to 750 bp) that lacked the Stat-1 binding region. These results indicated Stat-1 participation in IFN- γ stimulated OIP-1 gene expression in OCL precursor cells.

3.3. Stat-1 activation in RAW 264.7 cells

We further analyzed the specificity of Stat-1 activation and nuclear localization in RAW 264.7 macrophage cells in response to IFN- γ treatment. Immuno-cytochemical staining using anti-phospho Stat-1 antibody demonstrated nuclear localization of pStat-1 in RAW 264.7 cells in response to IFN- γ treatment. In contrast, unstimulated control RAW 264.7 cells did not demonstrate Stat-1 activation in these cells (Fig. 4A). Western blot analysis using anti-phospho Stat-1 antibody further confirmed high levels of phospho-Stat-1 (pStat-1) expression in total cell lysates derived from IFN- γ stimulated RAW 264.7 cells compared to unstimulated cells (Fig. 4B). However, we did not detect significant changes in phospho-Stat-3 levels in these cells in response to IFN- γ stimulation. These data further confirmed Stat-1 activation and its participation in IFN- γ induced OIP-1 gene expression in OCL precursor cells.

3.4. Stat-1 binding to OIP-1 gene promoter sequence

We also examined the status of Stat-1 binding to OIP-1 gene promoter element in response to IFN- γ treatment to OCL precursor cells. As evidenced by electrophoretic mobility shift assay (EMSA) analysis, nuclear extracts derived from IFN- γ stimulated RAW 264.7 cells demonstrated increased levels of activated Stat-1 binding to the OIP-1 gene promoter sequence compared to the unstimulated control cells. Antiphospho-Stat-1 antibody addition to the EMSA reaction decreased Stat-1 binding to the OIP-1 gene promoter region. Addition of excess cold probe ($\times$ 100) blocked Stat-1 binding to the DNA probe confirms the specificity of EMSA (data not shown). Furthermore, nuclear extracts derived from Stat-1 specific inhibitor, fludarabine (50 μ M) treated (24 h) IFN- γ stimulated RAW 264.7 cells did not demonstrate Stat-1 binding to the OIP-1 gene promoter sequence (Fig. 5A). Lack of binding to a mutant Stat-1 element (Mut-oligo) confirms the specificity of Stat-1 binding to the DNA probe used in the EMSA. We have further confirmed Stat-1 binding to OIP-1 gene promoter sequence by

oligonucleotide pull-down assay as described (Ikeda et al., 2004). As shown in Fig. 5B nuclear extracts (NE) prepared from the mouse bone marrow derived preosteoclast cells stimulated with IFN- γ for 30 min demonstrated p-Stat-1 affinity binding with biotinylated oligonucleotides containing the intact Stat-1 binding sequence present in OIP1 gene promoter. In contrast, a mutant oligo (Mut-oligo) which lacks Stat-1 recognition site did not demonstrate affinity binding to p-Stat-1. These data confirms the specificity of Stat-1 binding to OIP-1 promoter sequence and further suggests that IFN- γ regulates OIP-1 gene promoter activity in OCL precursor cells through a Stat-1 dependent signaling pathway.

3.5. TRADD negatively regulates OIP-1 gene promoter activity

Recent studies indicated that the TNF- α receptor interacting adaptor protein TRADD has been shown to influence IFN- γ signaling through interaction with Stat-1 alpha transcription factor (Wesemann et al., 2004). Therefore, we further examined if suppression of TRADD expression in OCL precursor cells affect IFN- γ signaling to enhance OIP-1 gene promoter activity. We have used siRNA interference method to suppress TRADD expression in OCL precursor cells as described in the methods. RAW264.7 cells were co-transfected with OIP-1 pGL2 Basic#3 luciferase reporter plasmid and siRNA for TRADD and measured the luciferase activity levels in response to IFN- γ treatment. As shown in Fig. 6A, siRNA suppression of TRADD results in significant (2.5 fold) increase in IFN- γ stimulation of OIP-1 gene promoter activity in RAW264.7 cells compared to IFN- γ treatment alone. Western blot analysis further confirmed siRNA suppression of TRADD expression and increased levels of phospho-Stat-1 (pStat-1) in response to IFN- γ stimulation (Fig. 6B). These results indicate that knockdown of TRADD enhances IFN- γ signaling to increase OIP-1 gene expression in OCL precursor cells.

3.6. IFN- γ regulation of OIP-1 and TRADD expression in mouse bone marrow derived preosteoclast cells

Since RAW 264.7 cells demonstrate a transformed phenotype, we further confirm IFN- γ regulation of OIP-1 and TRADD expression in mouse bone marrow derived preosteoclast cells. As shown in Fig. 7A real-time RT-PCR analysis of total RNA isolated from the mouse bone marrow derived preosteoclast cells stimulated with IFN- γ for indicated period (0–48 h) demonstrated a significant increase in the levels of OIP-1 mRNA expression. IFN- γ stimulation for 48 h period resulted in 11-fold increase in the levels of OIP-1 mRNA expression in preosteoclast cells. The relative levels of OIP-1 mRNA expression in these cells were normalized with respect to the levels of GAPDH amplification. We have also further confirmed IFN- γ induced expression of TRADD in mouse bone marrow derived preosteoclast cells. As shown in Fig. 7B Western blot analysis of total cell lysates obtained from the preosteoclast cells stimulated with IFN- γ for the indicated period demonstrated induced expression of TRADD in these cells. IFN- γ stimulation for 48 h resulted in a 7-fold increase in the levels of TRADD expression in preosteoclast cells. These data further confirms IFN- γ signaling to induce OIP-1 and TRADD expression in primary preosteoclast cells.

4. Discussion

Osteoclast formation and bone resorption activity are regulated by the local factors produced in the bone marrow microenvironment. Interferons, which are released in response to inflammatory stimuli, have been reported as important local negative regulators for bone remodeling (Roodman, 1999). Tumor necrosis factor (TNF) gene family member, receptor activator of NF- κ B (RANK) expressed on OCL precursors and RANK ligand (RANKL) expressed on osteoblast/stromal cells interaction is critical for OCL differentiation and bone resorption (Suda et al., 1999). 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 (Hsu et al., 1999). Recently

it has been shown that T-cell production of IFN- γ strongly suppresses osteoclastogenesis by rapid degradation of the TRAF-6 which results in strong inhibition of RANKL induced NF- κ B activation and p-c-Jun kinase activity (Takayanagi et al., 2000). These studies suggest that there is cross-talk between the TNF and IFN families of cytokines, through which IFN- γ provides a negative link between T-cell activation and bone resorption. IFN- γ exerts a direct effect on OCL progenitors and TGF β ₁ antagonizes the effect of IFN- γ (Fox and Chambers, 2000). OCL itself is not classified as an immune cell, however, it has been shown to produce factors such as interferon beta and tumor necrosis factor alpha which regulate OCL formation and bone resorption activity. It has also been shown that RANKL induces the interferon- β gene in OCL precursor cells, and that IFN- β inhibits OCL differentiation by interfering with the RANKL induced expression of *c-fos* to maintain bone homeostasis (Takayanagi et al., 2002). However, RANKL treatment also induces suppressors of cytokine signaling, SOCS-1 and 3 which suppress IFN signaling in OCL precursors counteracting IFN- β intrinsic inhibition of OCL development (Hayashi et al., 2002). Therefore, it is important to delineate the molecular mechanisms by which IFN- γ regulate gene expression in OCL precursor cells.

We have recently identified OIP-1/hSca, a potent inhibitor of osteoclastogenesis is produced by OCL precursor cells and upregulated in response to IFN- γ treatment (Koide et al., 2003). In the present study, isolation and sequence analysis of OIP-1/hSca gene promoter region identified several potential regulatory elements that are response to osteotropic factors (Fig. 2). Our results indicate the presence of a functional Stat-1 binding motif in the OIP-1 gene promoter region. Stat-1 specific inhibitor, fludarabine abolished IFN- γ stimulated OIP-1 gene promoter activity suggest that IFN- γ regulates OIP-1 gene promoter activity in OCL precursor cells through a Stat-1 dependent signaling pathway. This is in contrast to IL-4, which abrogates osteoclastogenesis through STAT6-dependent inhibition of NF- κ B (Abu-Amer, 2001). Furthermore, it has been shown that IFN- γ inhibit 1,25-(OH)₂D₃ induced tartrate resistant acid phosphatase (TRAP) positive multinucleated cell (MNC) formation in long-term human bone marrow cultures. In addition, IFNs inhibit GM-CFU (granulocyte-macrophage colony-forming unit) growth and the ability of OCL precursors to fuse and form multinucleated OCLs (Roodman, 1999). Therefore, IFN- γ regulation of OIP-1 gene expression may be complex and multifactorial. This could be further limited if the stability of the OIP-1 mRNA is altered by IFN- γ stimulation. However, in the present study, identification of Stat-1 functional motif may provide molecular insights into OIP-1 gene regulation with respect to heteromeric/multifactorial events associated with the OIP-1 gene expression in normal and pathological conditions. Recent evidence suggests that TRADD negatively regulates the IFN- γ signaling (Wesemann et al., 2004). Our data indicating significant increase in IFN- γ stimulated OIP-1 gene promoter activity upon suppression of TRADD expression in RAW264.7 cells further suggests the participation of TRADD and negative regulation of IFN- γ signaling in OCL precursor cells. However, the moderate increase observed in OIP-1 gene promoter activity by TRADD siRNA interference alone could be due to constitutive interaction of TRADD and background levels of activated STAT-1 in these transformed cells. Therefore, these results should provide insights into the molecular control of OIP-1 gene expression and inhibition of OCL activity in the bone microenvironment.

Abbreviations

OCL, osteoclast; OIP-1/hSca, osteoclast inhibitory peptide-1; Stat-1, signal transducer and activator of transcription-1; EMSA, electrophoretic gel mobility shift assay; BAC, bacterial artificial chromosome; IFN- γ , interferon gamma; RANKL, receptor activator of NF- κ B ligand; TRADD, tumor necrosis factor receptor 1 associated death domain protein.

Acknowledgments

This work was supported by the National Institute of Health grants DE 12603 and Department of Defense Medical Research Award.

References

- Abu-Amer Y. IL-4 abrogates osteoclastogenesis through STAT6-dependent inhibition of NF-kappaB. *J. Clin. Invest* 2001;107:1375–1385. [PubMed: 11390419]
- Bonyadi M, Waldman SD, Liu D, Aubin JE, Grynblas MD, Stanford WL. Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. *Proc. Natl. Acad. Sci. U. S. A* 2003;100:5840–5845. [PubMed: 12732718]
- Fox SW, Chambers TJ. Interferon-gamma directly inhibits TRANCE-induced osteoclastogenesis. *Biochem. Biophys. Res. Commun* 2000;276:868–872. [PubMed: 11027561]
- Hayashi T, Kaneda T, Toyama Y, Kumegawa M, Hakeda Y. Regulation of receptor activator of NF-kappa B ligand-induced osteoclastogenesis by endogenous interferon-beta (INF- β) and suppressors of cytokine signaling (SOCS). The possible counteracting role of SOCSs in IFN- β -inhibited osteoclast formation. *J. Biol. Chem* 2002;277:27880–27886. [PubMed: 12023971]
- Hoshika S, Minakawa N, Kamiya H, Harashima H, Matsuda A. RNA interference induced by siRNAs modified with 4'-thioribonucleosides in cultured mammalian cells. *FEBS Lett* 2005;579:3115–3118. [PubMed: 15919084]
- Hsu H, et al. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc. Natl. Acad. Sci. U. S. A* 1999;96:3540–3545. [PubMed: 10097072]
- Ikeda F, et al. Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. *J. Clin. Invest* 2004;114:475–484. [PubMed: 15314684]
- Khan M, et al. Administration of N-acetylcysteine after focal cerebral ischemia protects brain and reduces inflammation in a rat model of experimental stroke. *J. Neurosci. Res* 2004;76:519–527. [PubMed: 15114624]
- Kim U, et al. Construction and characterization of a human bacterial artificial chromosome library. *Genomics* 1996;34:213–218. [PubMed: 8661051]
- Koide M, Kurihara N, Maeda H, Reddy SV. Identification of the functional domain of osteoclast inhibitory peptide-1/hSca. *J. Bone Miner. Res* 2002;17:111–118. [PubMed: 11771657]
- Koide M, Maeda H, Roccisana JL, Kawanabe N, Reddy SV. Cytokine regulation and the signaling mechanism of osteoclast inhibitory peptide-1 (OIP-1/hSca) to inhibit osteoclast formation. *J. Bone Miner. Res* 2003;18:458–465. [PubMed: 12619930]
- Kosugi A, Saitoh S, Narumiya S, Miyake K, Hamaoka T. Activation-induced expression of thymic shared antigen-1 on T lymphocytes and its inhibitory role for TCR-mediated IL-2 production. *Int. Immunol* 1994;6:1967–1976. [PubMed: 7696214]
- Kosugi A, et al. Physical and functional association between thymic shared antigen-1/stem cell antigen-2 and the T cell receptor complex. *J. Biol. Chem* 1998;273:12301–12306. [PubMed: 9575182]
- MacNeil I, et al. Isolation of a cDNA encoding thymic shared antigen-1. A new member of the Ly6 family with a possible role in T cell development. *J. Immunol* 1993;151:6913–6923. [PubMed: 8258699]
- Mao M, et al. RIG-E, a human homolog of the murine Ly-6 family, is induced by retinoic acid during the differentiation of acute promyelocytic leukemia cell. *Proc. Natl. Acad. Sci. U. S. A* 1996;93:5910–5914. [PubMed: 8650192]
- Noda S, Kosugi A, Saitoh S, Narumiya S, Hamaoka T. Protection from anti-TCR/CD3-induced apoptosis in immature thymocytes by a signal through thymic shared antigen-1/stem cell antigen-2. *J. Exp. Med* 1996;183:2355–2360. [PubMed: 8642345]
- Ramana CV, Gil MP, Schreiber RD, Stark GR. Stat-1 dependent and independent pathways in IFN- γ dependent signaling. *Trends Immunol* 2002;23:96–101. [PubMed: 11929133]
- Roodman GD. Cell biology of the osteoclast. *Exp. Hematol* 1999;27:1229–1241. [PubMed: 10428500]
- Saitoh S, et al. Modulation of TCR-mediated signaling pathway by thymic shared antigen-1 (TSA-1)/stem cell antigen-2 (Sca-2). *J. Immunol* 1995;155:5574–5581. [PubMed: 7499840]

- Shan X, et al. Characterization and mapping to human chromosome 8q24.3 of Ly-6-related gene 9804 encoding an apparent homologue of mouse TSA-1. *J. Immunol* 1998;160:197–208. [PubMed: 9551972]
- Stefanova I, Horejsi V, Ansotegui JJ, Knapp W, Stockinger H. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* 1991;254:1016–1019. [PubMed: 1719635]
- Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr. Rev* 1999;20:345–357. [PubMed: 10368775]
- Takayanagi H, et al. T-cell-mediated regulation of osteoclastogenesis by signaling cross-talk between RANKL and IFN-gamma. *Nature* 2000;408:600–605. [PubMed: 11117749]
- Takayanagi H, et al. RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. *Nature* 2002;416:744–749. [PubMed: 11961557]
- Wada T, et al. The molecular scaffold Gab2 is a crucial component of RANK signaling and osteoclastogenesis. *Nat. Med* 2005;11:394–399. [PubMed: 15750601]
- Wesemann DR, Qin H, Kokorina N, Benveniste EN. TRADD interacts with STAT-1- α and influences interferon- γ signaling. *Nat. Immunol* 2004;5:199–207. [PubMed: 14730360]

Southern Blot Analysis of OIP-1 BAC Genomic Clone

Bst x1

Partial Restriction Enzyme Map

Fig. 1.

Isolation and partial restriction map of the OIP-1 gene promoter region. Southern blot analysis of the BAC genomic clone (314A1) encoding the human OIP-1 gene (*left panel*). Partial restriction enzyme map of the 5'-flanking region of the human OIP-1 gene marked with a 4 Kb DNA fragment flanked by Bst $\times$ 1 restriction enzyme sites (*Right panel*).

cccacccct tccgggagac ggcgcctgt gcctctcgcg tgtatccatt tataattaac -2261
tgcaaatgtg tttccctgag ttccctgagc cactcttgca aatcaattga acgcaaaacc -2201
GC-box
 gggaggggct gcagtattgc tgggaaagag ggtcctgatc cagaccccaa gagcagggtc -2141
 ttggatgttg tgcaggaagt aatttgggt gagccacaga gcacagttag agaagcagggt -2081
 ttatttgaaa tggctccgtt ccagagtggg SP-1 gcgtgctcag aaagcaggag gaggaggggc -2021
 tgtcctctgt tagtgctctt gcttacaaga aactgtaagg agctacaatt acacctggaa -1961
 tgggcagatg cacccaactaa GATA-1, 2, 3 ggataggggc tgtgggtgcc atcagtggcc gttcatcctc -1901
 caacctcagg ctgctcattg aaattatctc tgagtaaagt ggctgcact Nkx-2 cttgggttat -1841
 ctggacagtc tgcgggcttg ctgggagatg GATA-1, 2 SRF ccacaaatat CdxA tctgtaatta -1781
taattgtgtg ccagcttagg acatggctat RSRFC4 ttttagacca taagcattaa ccttataggt -1721
 gccttgtgag tgcctctac tcaactcaag GATA-1 atggagtcac tctggtcatg tttcattaaa -1661
 ccagaggcct ggtgagcaag Stat-1 cgttcctcta acagtgggaa cctcaacgtc gggccagtcg -1601
 gtaagaagt ccagaggctg gccctcgcag ctcatgtgtg tgcatatgtg tgtgtgtgtg -1541
 tgtgtgtgtg tgtgtgtgta ggtgtctgga tctgacgcta tctgcaggta Elf-1 cggaatggt -1481
gttggaggac AP-4 actcggctgg tgtccgctgc agaactgatg gcttgcctgg SP-1 tgccggggag -1421
acgtccacct MyoD gccacatttg gccccagcag tcttgggagt ctctgtgtt gatgtcttct -1361
 gtgttgagt agagacagga GATA-1 taaatggtt gagagtttt tccaaacagc cctcctccta -1301
 atgcagcttg ttttctgaat AP-1 cagagggctc tgtcttgaag ggggcaaacc caccgtgct -1241
 ttcgtccac agttcttact gagccctgc catggcagg GATA-1 atggccccag gtgcccgtg -1181
 cagagacaga accccctgcc ctcaccgcca acattccaga ggggaagatg ccctgcctgt -1121
 gccccacgta agacccagag NF-kap gcagggaactc ccccgggcca cagcctactg gaggtcctg -1061
 tccccagaga AP-4 cagctgggcc atcgccccca aagctgtgga agccccacc acgatgccat -1001
 cataccacgg ccaggacatc tcgtgacaca cacagcccca gcctcagcct ccatacacac -941
 acacacacgc accacacaca ctcactcatg Brn-2 caaaacacac acacagccac attgactgca -881
 cccctagatt tgagacaggg gtgttacagg tgtctgtcag cccagcccc ctctgacca -821
 catggccacc Myo-D tgccaccct cacatttccg cacaccctcc cctactccc gtgtggggtt -761
 cagaagacc ctccctgtg ctgcctgtg caccagaaa ISRE ggaaacgcac c-Myb tgtcaactgg -701
 agaacaggcc ggtggcagag cccagcgcc cacagccct cggagagggc ctggcgggag -641
 gagaggcacc caccgatggc agagcctcag tgcctcgcga gaagccagca cccacaacct -581
 cccacctccc aacccttgc ggtctcccca ccccgctg GATA-1, 2 agggatggcc aagggaacagc -521
ccagccctgga AP-4 c-Ets- gcgggaaagca gggacaagat gacctcagga atcccaggct gcgggggtgc -461
 ctgcgtcctc ccctcccgag ctccactccg caggcgagac ccccaacact gctccccgac -401
 actctccgag gcacccctcc tccgtgctca caccacaaaca cgtctgctca cccgtgggct -341
 tcccaccccc aacacaacga ggactgtcca ggtgtcagtg acggtcacc cgcctgtgc -281
 ccctgtccgg cctcgccagg MyoD tcagcacccg AP-4 ccgcgcgcac agcagggccg ggtggaagga -221
 ggccgggctt ggccgtggct deltaE caggcgccac ctcaactggg aaactgggag gtggtctcag -161
 ccccttctg ggccctcagct tcccacatca tcccaaggag ccggggtagg cctgggcggc -101
gcggggaccc NF-kap ccaccgcggg ctgcgcgact ccgccgcgc gagggccctc ccgcgtcccg -41
 cccctgcccc cgcgccgggg ccgcgcgggt gcctgggagg ctccggggcca gccgcgggtcc +20
 +1

Fig. 2. Nucleotide sequence analysis of OIP-1/hSca gene promoter region. Transcription factor binding motifs with significant identity (>85%) in the OIP-1 gene promoter region are underlined. Transcription start site is noted as +1.

Fig. 3.

IFN- γ stimulation of OIP-1 gene promoter activity. (A) OIP-1 pGL2Basic#3 promoter-luciferase reporter plasmid was transiently transfected into RAW cells (5×10^5 cells/well) in a six well plate. The cells were stimulated with IFN- γ (50 ng/ml) for 48 h and assayed for luciferase activity in the total cell lysates. pGL2 basic vector transfected cells were served as control. (B). Effect of Stat-1 inhibitor on IFN- γ stimulated OIP-1 gene promoter activity. OIP-1 pGL2Basic#3 plasmid DNA (2 μ g) was transiently transfected into RAW 264.7 cells by Lipofectamine (Invitrogen Inc., Carlsbad, CA) method and cultured in the presence and absence of IFN- γ (50 ng/ml). Stat-1 specific inhibitor, fludarabine (50 μ M) was treated to the cells for 24 h prior to IFN- γ stimulation. Total cell lysate prepared after 48 h were assayed for luciferase activity. Transfection efficiency was normalized by measuring β -galactosidase activity co-expressed in these cells ($P>0.05$).

Fig. 4. Phospho-Stat-1 (pStat-1) expression in RAW 264.7 cells. (A) Nuclear localization of pStat-1 in RAW 264.7 cells. The cells were stimulated with IFN- γ (50 ng/ml) for 30 min period and immunocytochemical staining was performed using anti-pStat-1 antibodies as described. Nuclear localization of p-Stat-1 in IFN stimulated cells is pointed by an arrow. (B) Western blot analysis of pStat-1 expression in RAW 264.7 cell lysates. RAW 264.7 cells were seeded in 10 cm plates at a density of 10^6 cells in 10 ml of α -MEM medium containing 10% fetal calf serum and cultured for 24 h in the presence of Stat-1 specific inhibitor, fludarabine (50 μ M) prior to IFN- γ (50 ng/ml) stimulation for 30 min and total cell lysates obtained were subjected to Western blot analysis as described in methods.

Fig. 5.

Stat-1 binding to the OIP-1 gene promoter sequence. (A) Nuclear extracts (NE) were prepared from RAW 264.7 cells stimulated with IFN- γ (50 ng/ml) for 30 min and EMSA analysis was performed with 32 P radiolabelled Stat-1 binding element present in OIP-1 promoter sequence as described in methods and electrophoresed on 5% native polyacrylamide gels. A control oligomeric probe in which Stat-1 element was mutated (Mut-oligo) demonstrate no significant mobility shift confirms the specificity of Stat-1 binding to DNA probe. (B) Oligonucleotide pull-down assay for Stat-1 binding to OIP-1 gene promoter sequence. NE were prepared from the mouse bone marrow derived non-adherent cells cultured in the presence of M-CSF for 24 h period (preosteoclast cells) as described (Wada et al., 2005) and stimulated with and without

IFN- γ for 30 min. (a) The NE were incubated for 2 h with biotinylated oligonucleotides containing the intact Stat-1 binding sequence present in OIP1 gene promoter and Mut-oligo; and analyzed by immunoblotting with anti-pStat-1 antibody. (b) Stat-1 and pStat-1 expression in the NE was also confirmed by Western blot analysis as described in methods.

Fig. 6.

Effect of TRADD on OIP-1 gene promoter. (A) IFN- γ (50 ng/ml) stimulation of OIP-1 gene promoter activity in the presence and absence of TRADD siRNA in RAW264.7 cells. TRADD siRNA and OIP-I pGL2 basic#3 plasmid DNA were transiently transfected into RAW264.7 cells by Lipofectamine method. After 48 h, cells were incubated with serum free medium with or without IFN- γ (50 ng/ml) for 2 h period. Total cell lysates obtained were assayed for luciferase activity. Transfection efficiency was normalized by measuring β -galactosidase activity co-expressed in these cells ($P>0.05$). (B) Western blot analysis for TRADD and pStat-1 expression in RAW264.7 cells in response to IFN- γ (50 ng/ml) treatment and TRADD siRNA interference.

Fig. 7. IFN- γ regulation of OIP-1 and TRAD expression in mouse bone marrow derived preosteoclast cells. (A) Quantitative real-time RT-PCR analysis of OIP-1 mRNA expression in mouse bone marrow derived preosteoclast cells. The cells were stimulated with IFN- γ for indicated period (0–48 h) and total RNA isolated was subjected to real-time RT-PCR analysis as described in methods. Relative levels of OIP-1 mRNA expression were normalized with respect to the levels of GAPDH amplification. (B) Non-adherent mouse bone marrow cells stimulated with M-CSF for 24 h. The resultant cells (preosteoclast cells) were stimulated with IFN- γ for indicated time points. Total cell lysates obtained from these preosteoclast cells were subjected to Western blot analysis of TRADD expression.