Journal of Cellular Biochemistry 84:645–654 (2002)

Characterization of Interferon Gamma Receptors on Osteoclasts: Effect of Interferon Gamma on Osteoclastic Superoxide Generation

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Abstract Osteoclasts are the primary cells responsible for bone resorption. Osteoclast formation and bone resorption activities involve processes tightly controlled by a network of cytokines. The presence of interferon gamma (IFN-γ) receptors on osteoclasts is a necessary prerequisite for IFN-γ to directly affect osteoclastic activity. To date, the presence of the IFN-γ receptor on osteoclasts has not been established. This study provides evidence that osteoclasts express the IFN-γ receptor. Specific binding of IFN-γ to the osteoclastic receptor stimulates osteoclastic superoxide generation. The p91 and p47 components of the NADPH oxidase increase after IFN-γ stimulation and may account for the enhanced superoxide generation. Antisense experiments targeting p91 and p47 subunits abrogate the increased osteoclastic superoxide production stimulated by IFN-γ. Thus, superoxide generation by osteoclasts is stimulated by activation of a functional IFN-γ receptor on the osteoclast. J. Cell. Biochem. 84: 645–654, 2002.

Key words: osteoclast; interferon gamma; receptor; superoxide; NADPH oxidase

Interferon γ (IFN-γ) is a cytokine produced by T lymphocytes and natural killer cells. IFN-γ is necessary for differentiation, development, and function of the immune system. Many cells (T-/B-lymphocytes, macrophages, monocytes, and neutrophils) express IFN-γ receptors and respond to IFN-γ stimulation. Upon binding to a specific cell surface receptor, IFN-γ induces antiviral activity, upregulating major histocompatibility complex (MHC) expression, B cell maturation, activation of cells to cytotoxic states, and release of mediators of inflammation [Friedman and Vogel, 1983]. IFN-γ stimulation of superoxide production appears to induce NADPH oxidase gene expression [Cassatella et al., 1990; Gupta et al., 1992]. The resultant effect of IFN-γ stimulation of neutrophils is an enhanced capacity to generate superoxide in response to bacterial/viral stimuli. In addition, NADPH oxidase has been demonstrated to be an enzyme system contributing to osteoclastic superoxide generation [Steinbeck et al., 1994; Darden et al., 1996; Yang et al., 1998].

The IFN-γ receptor consists of two subunits, a 90-kDa α-chain and a 36-kDa β-chain [Schreiber et al., 1992]. The IFN-γ receptor α-chain was demonstrated to have an extracellular domain, transmembrane section, and cytoplasmic domain [Aguet et al., 1988]. The extracellular domain of IFN-γ receptors exhibits a high affinity ligand binding property and is responsible for IFN-γ binding. The β-chain of the IFN-γ receptor is required primarily for signaling [Farrar and Schreiber, 1993].

Osteoclasts have been demonstrated to express the calcitonin receptor [Nicholson et al., 1986], the M-CSF receptor [Hofstetter et al., 1992; Yang et al., 1996], the estrogen receptor [Pederson et al., 1997], the IL-6 receptor [Ohsaki et al., 1992], and the RANKL (receptor activator of NF-κB ligand) receptor [Burgess et al., 1999; Wong et al., 1999]. The presence of these receptors on osteoclasts directly regulates the function and activity of osteoclasts. In this study, we demonstrated that osteoclasts express the IFN-γ receptor and respond to

Grant sponsor: URC in Medical University of South Carolina; Grant sponsor: General Clinical Research Center at Medical University of South Carolina.

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Received 2 July 2001; Accepted 12 October 2001

DOI 10.1002/jcb.10074

stimulation by binding to IFN-γ, enhancing production of superoxide.

MATERIALS AND METHODS

^{125}I-labeled IFN-γ (2,300 Ci/mmol) was purchased from NEN Life Science Product (Boston, MA). Recombinant human IFN-γ (3×10^4 U/μg) was obtained from Genentech, Inc (San Francisco, CA). Anti-human IFN-γ receptor α chain antibody was purchased from Calbiochem (San Diego, CA). Anti-mouse IFN-γ receptor α chain antibody was obtained from BD Biosciences (San Diego, CA). Goat anti-p47 antibody was a generous gift from Dr. Thomas Leto (National Institutes of Health, Bethesda, MD). RANKL was a generous gift from Amgen (Thousand Oaks, CA). RNAzol B was purchased from TEL-TEST, INC. (Friendswood, TX). Phosphorothioated sense and antisense oligonucleotides were synthesized and HPLC purified by Intergrated DNA technologies, Inc (Coralville, IA).

Osteoclast Culture

Human osteoclasts were obtained by culture of white blood cells as described [Matayoshi et al., 1996; Matsuzaki et al., 1998]. In brief, a large amount of mononuclear cells were obtained by leukapheresis. The cells were washed twice and resuspended in α-MEM medium containing FBS (10%), RANKL (50–100 ng/ml), and M-CSF (25 ng/ml). The cells were then cultured in 24-well plates (3×10^6 cells/well) or 10-mm culture dishes (5×10^7 cells/dish) for 1–2 weeks. The medium was changed twice a week by replacing half volume of medium with fresh medium. At the end of culture, contaminating cells were removed by a brief digestion (5–10 min) with lipase and collagenase (1 mg/ml) followed by vigorous washings. Approximately 10,000–100,000 osteoclasts can be generated in the culture. This cell population was 90% pure osteoclasts. These osteoclasts stained positively with TRAP (tartrate resistant acid phosphatase) and were able to make pits on bovine bone slices [Madyastha et al., 2000].

Osteoclast Micromanipulation

Culture dishes containing osteoclasts were washed with PBS to remove non-adherent cells. Individual osteoclasts were harvested by micromanipulation [Tong et al., 1994]. Briefly, a siliconized micropipette was connected with a suction tube and mounted on a micromanipulator. With the aid of microscope, osteoclasts were identified by their multiple nuclei, pseudopodia, and brush-like borders and were selectively harvested by suction into a micropipette. Micromanipulated osteoclasts were immediately transferred to a tube for RNA extraction.

Immunostaining

Similar osteoclasts were generated in chamber slides as described above. After removing nonadherent cells by vigorous washings, osteoclasts were fixed with 10% formalin for 10 min at room temperature, then washed three times with 1 × PBS. Hydrogen peroxide (3%) was applied to the slides and incubated for 5 min. After rinsing twice with PBS, slides were blocked using 1% BSA in 0.05 M Tris-HCl buffer, pH 7.5 for 10 min. Slides then were incubated with the IFN-γ receptor antibody (0.1 μg/ml) diluted with 1% BSA in 0.05 M Tris-HCl buffer, pH 7.5 for 10 min. After washing twice, a biotinylated link antibody was applied to the slides and incubated for 10 min. Streptavidin conjugated horseradish peroxidase then was added to the slides and incubated for 10 min. Finally, a DAB (diaminobenzidine) substrate solution was applied to the slides to visualize the bound antibody. The same isotype of immunoglobulin (IgG_1) was included as a control.

Western Blot Analysis

Osteoclasts were cultured in 10-cm plastic culture dishes as described above. After vigorous washings with 1 × PBS, non-osteoclastic cells were removed after a brief digestion with collagenase/dispase (1mg/ml) for 5–10 min. The remaining attached cells were 90% pure osteoclasts. These osteoclasts were incubated with 2 ng/ml of IFN-γ for 2 days. Thereafter, the osteoclasts were lysed with the RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 1% Triton X 100, 0.5% NP 40, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μM benzamidine). Osteoclast lysates were collected and incubated for 30 min at 4°C. The protein concentration was determined by the BCA (bicinchoninic acid) reagent. The lysates (50 μg) were loaded on a 10% SDS acrylamide gel. After transfer to a PVDF membrane, the membrane was incubated with IFN-γ receptor antibody (0.1 μg/ml). HRP-conjugated secondary antibody (1:5,000) was used to visualize the bound antibody by ECL (Amersham, Piscataway, NJ).

IFN-γ Receptor Binding on Osteoclasts

Osteoclasts were cultured in chamber slides as described above. After removing nonadherent cells by vigorous washings, osteoclasts were fixed with 10% formalin for 10 min. After washing twice with 1 × PBS, slides were stained with TRAP to identify multinuclear osteoclasts. Slides then were incubated with 0.3 ml of ^{125}I labeled IFN-γ (50,000 cpm) diluted with α-MEM medium containing 1% BSA for 2–3 h at room temperature. Nonspecific binding was assessed in the presence of 1 μg/ml of unlabeled IFN-γ. At the end of incubation, slides were washed three times with PBS and fixed for 10 min at room temperature in a 0.1 M sodium cacodylate buffer containing 2% formaldehyde and 2% glutaraldehyde. Thereafter, slides were washed once with 0.1 M of sodium cacodylate buffer. After air drying, slides were dipped in NTB2 liquid photographic emulsion (Kodak, Rochester, NY) and exposed in a light-tight box at 4°C for 3–4 weeks. Slides then were developed in D-19 solution and fixed. The autoradiograms of IFN-γ ligand binding were examined under a microscope.

Quantitative Analysis of the INF-γ Receptor

Scatchard analysis were performed to illustrate the receptor capacity and the affinity [Yang et al., 1992]. Human osteoclast-like cells FLG 29.1 were washed with HBSS and resuspended in HBSS at 1×10^7/ml. The receptor binding was performed in 100 μl containing 1×10^6 cells, 100,000 cpm ^{125}I-labeled INF-γ, and serious amounts of unlabeled INF-γ (20–15,000 pM). Non-specific binding was assessed by incubation of cells with 0.2 μg/ml of unlabeled INF-γ. Cells were incubated at room temperature for 3 h. The receptor binding was stopped by adding 1 ml of washing solution (0.1% BSA in HBSS). Cell pellets were washed twice and counted. The specific binding was obtained by subtracting the non-specific binding (^{125}I-labeled INF-γ plus unlabeled INF-γ) from a total binding (^{125}I-labeled INF-γ only) and was used for Scatchard analysis by the ligand program (Biosoft).

Superoxide Generation

Osteoclastic superoxide generation was determined by the NBT (nitroblue tetrazolium) reduction assay [Key et al., 1990]. Briefly, osteoclasts were cultured in the chamber slide for 10 days. After removing non-osteoclast cells by collagenase/dispase (1 mg/ml) digestion and vigorous washings, the remaining osteoclasts were incubated with IFN-γ (2 ng/ml) for 2 days. Then osteoclasts were incubated with the NBT solution containing 2.0 mg/ml of NBT and 35% FBS in RPMI 1640 medium for 1 h at 37°C. After incubation, osteoclasts were fixed in 10% formalin. The NBT staining intensity of individual osteoclasts was measured and quantified by microdensitomety using an inverted microscope (Olympus) equipped with a CCD camera. The camera image was displayed on a high-resolution monitor (Sony), stored in a microcomputer (ALR, Inc., Irvine, CA), and analyzed using densitometry software (Cue 2d, Opelco). Twenty randomly selected NBT-stained osteoclasts from each experiment were analyzed. The mean optical density was determined. The specificity of the NBT reduction assay for osteoclastic superoxide production has been demonstrated previously [Key et al., 1990; Ries et al., 1992].

For antisense experiments, osteoclasts were incubated with 2 ng/ml of IFN-γ along with 25 μM of antisense (p47-AS: 5′-CCAGCAGGGCGATGTGACGGATGAA-3′, p91-AS: 5′-TGCAATGGTGTGAATCGCA-3′) and corresponding sense (SS) oligonucleotides. After 2 days incubation, osteoclast viability was determined by exclusion of trypan blue from the cytoplasm. Osteoclastic superoxide generation was determined by the NBT method described above. The staining intensity was expressed as mean ± standard deviation and subjected to statistical analysis with a Student's *t*-test.

Quantitative PCR

Osteoclasts were cultured in 10-cm culture dishes. Nonadherent cells were removed by adding collagenase/dispase (1 mg/ml). After vigorous washings with sterile 1 × PBS, RNAzol B was added at 0.25 ml per dish. Cell lysates were collected. Total RNA was extracted. Reverse transcription was conducted using oligo dT primer. The PCR mixture was prepared as follows: 5 μl of the RT mixture, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 1.25 U AmpliTag, 25 pmol of gene specific primers for p91, and serial dilutions of competitive templates. Thirty-five cycles were run for 1 min at 94, 55, and 72°C. A 280-bp PCR product was generated from native p91 mRNA and a 380-bp fragment was obtained from a competitive

template. The densities of individual bands were determined by scanning and analyzed by densitometric software (NIH Image). The ratio of band density generated (380 bp/280 bp) was plotted against the concentrations of the competitive templates. The amount of p91 mRNA present in the sample was defined as the concentration of a competitive template where the ratio equals 1 [Gilliland et al., 1990; Vanden-Heuvel et al., 1993].

RESULTS

Expression and Binding Activity of the IFN-γ Receptor

Using the anti-IFN-γ receptor antibody, expression of the IFN-γ receptor was demonstrated on human osteoclasts generated in culture (Fig. 1A) and on mouse osteoclasts isolated from neonates (Fig. 1B). The osteoclasts were identified as multinuclear giant cells that were positively stained at both cell membrane and cytoplasm. This cell population was 90% pure osteoclasts (Fig. 1A). Contaminating cells were washed away. Additional contaminating cells were removed by a brief digestion with lipase and collagenase. Little or no staining for the IFN-γ receptor was seen when the same isotype of immunoglobulin (IgG_1) was included (data not shown). This result suggests the specific binding of the antibody to the osteoclastic receptor.

The response of a cell to IFN-γ stimulation requires not only expression of the receptor but also the capability of the receptor to bind IFN-γ. Thus, a receptor-binding assay was performed to determine the binding activity of the IFN-γ receptor in osteoclasts. As shown in Figure 1C, the exposed silver grains are more evenly distributed along the entire cell membrane compared with the control shown in Figure 1D. Binding of ^{125}I-labeled IFN-γ to osteoclasts can be competitively inhibited by an excess of unlabeled IFN-γ (1 μg/ml, Fig. 1D), however,

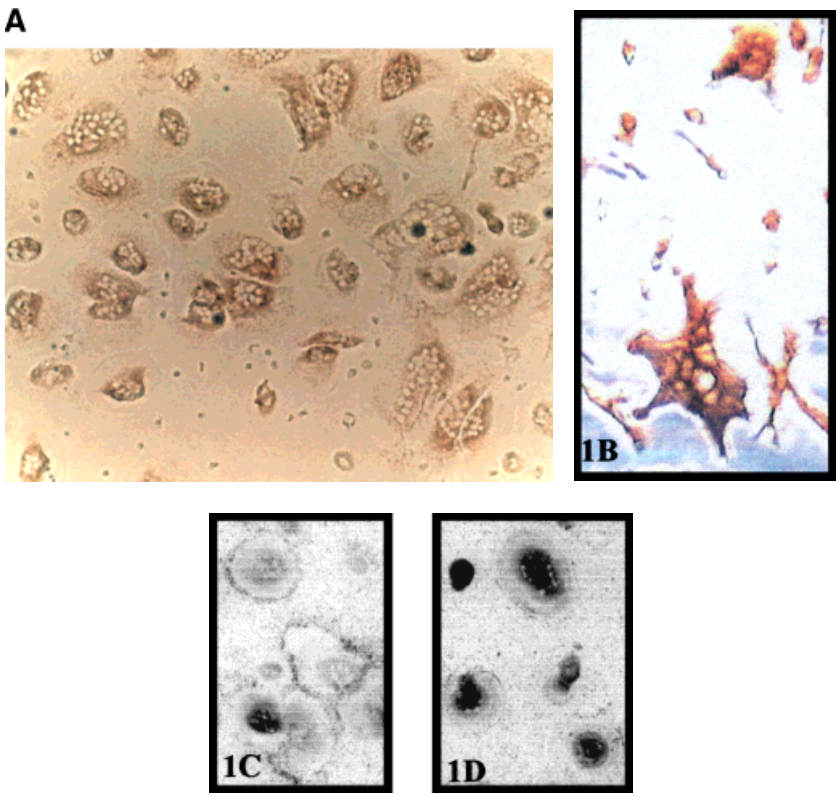

Fig. 1. Expression and binding activity of IFN-γ receptors. Osteoclasts were cultured in chamber slides as described in Materials and Methods. After fixing and blocking, the IFN-γ receptor antibody was added. The bound antibody was visualized by the DAKO LSAB System using DAB as a substrate-chromogen. **A**: Human osteoclasts were obtained by culture and stained by the IFN-γ receptor antibody (0.1 μg/ml). **B**: Mouse osteoclasts were isolated from C57Bl/6 neonates and stained by the IFN-γ receptor antibody (0.2 μg/ml). **C**: Binding of ^{125}I-labeled IFN-γ ligand to its receptor was demonstrated on osteoclasts. **D**: Binding was performed in the presence of ^{125}I-labeled and unlabeled IFN-γ to reveal non-specific binding on osteoclasts. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

positive binding grains were also observed when 1 μg/ml of unlabeled M-SCF was included (data not shown). It suggests the specificity of the IFN-γ binding to osteoclasts.

Quantitative Analysis of the INF-γ Receptor

Scatchard analysis was performed to illustrate the IFN-γ receptor density and affinity using the osteoclastic cell line FLG 29.1 which can be further differentiated into mature osteoclasts, capable of bone resorption activity [Gattei et al., 1992]. As shown in Figure 2A, Scatchard plot revealed a single class of the receptor binding sites of IFN-γ. The capacity of IFN-γ receptor in FLG 29.1 cells is ~6,200 receptor sites per cell and the affinity of IFN-γ receptor is about 1.9 nM. This is consistent with the reports observed on most cells [Farrar and Schreiber, 1993]. The binding of ^{125}I-labeled IFN-γ to the cellular receptor was reduced by 85% when unlabeled IFN-γ was present. However, unrelated cytokines (M-CSF, IL-1, IL-6) were unable to compete for binding with the IFN-γ (Fig. 2B). This suggests that only unlabeled IFN-γ inhibits ^{125}I-labeled IFN-γ binding, indicating the specificity of IFN-γ ligand binding in this study.

Downregulation of the IFN-γ Receptor

After stimulation with IFN-γ, the total amount of IFN-γ receptors on osteoclasts was assessed by Western blot analysis. As shown by Figure 3A, approximately 50% of IFN-γ receptor protein were reduced after IFN-γ stimulation. In addition, the IFN-γ receptor mRNA level was reduced by IFN-γ stimulation (Fig. 3B). This indicates that stimulation of osteoclasts by IFN-γ resulted in a decreased amount of the IFN-γ receptor present on osteoclasts, suggesting the IFN-γ ligand-induced receptor downregulation. The downregulation of IFN-γ receptor may associate with the apoptosis of osteoclasts.

Effect of IFN-γ on Osteoclastic Superoxide Generation

Superoxide production by individual osteoclast was examined by the NBT assay. Unlike the cytochrome C assay that measures changes in solution that could result from non-osteoclast cells present in the culture, the NBT assay directly measures changes in osteoclasts by the density of intracellular precipitates (diformazan). Thus, the NBT assay used in our experiment directly reflects changes in osteoclasts, not on other contaminated cells since their phenotypes can be easily distinguished under microscopy. Twenty osteoclasts for each group were randomly selected and the NBT staining densities were analyzed by microdensitometry. Osteoclastic superoxide generation was increased by 40% after the purified osteoclasts were stimulated with IFN-γ (Fig. 4). Since the receptors for interferon gamma were seen exclusively on osteoclasts, the stimulation appears to result from the interaction of IFN-γ with its osteoclastic receptor.

Effect of IFN-γ on NADPH-Oxidase Expression in Osteoclasts

To examine the effect of IFN-γ on expression of NADPH oxidase in osteoclasts, the relative amounts of p47 and p91 subunits in osteoclasts

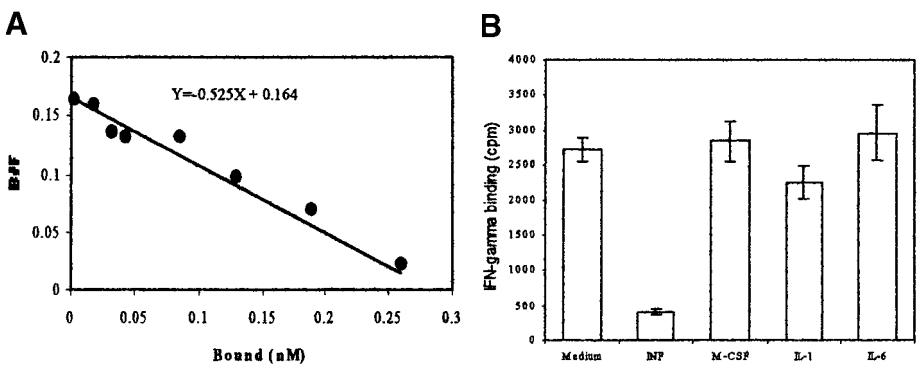

Fig. 2. Quantitative analysis of the INF-γ receptor. The receptor binding was performed in 100 μl containing 1×10^6 of human osteoclast-like cells, 100,000 cpm ^{125}I-labeled INF-γ, and serious amounts of unlabeled INF-γ (20–15,000 pM) as described in Materials and Methods. The specific binding was obtained by subtracting the non-specific binding (^{125}I-labeled INF-γ plus unlabeled INF-γ) from total binding (^{125}I-labeled INF-γ only). **A**: Scatchard analysis to illustrate the presence of ~6,200 receptor sites per cell with the affinity of 1.9 nM. **B**: Competitive binding of ^{125}I-labeled IFN-γ. Binding of ^{125}I-labeled IFN-γ was performed in the presence of indicated cytokines to illustrate the specificity of the receptor binding.

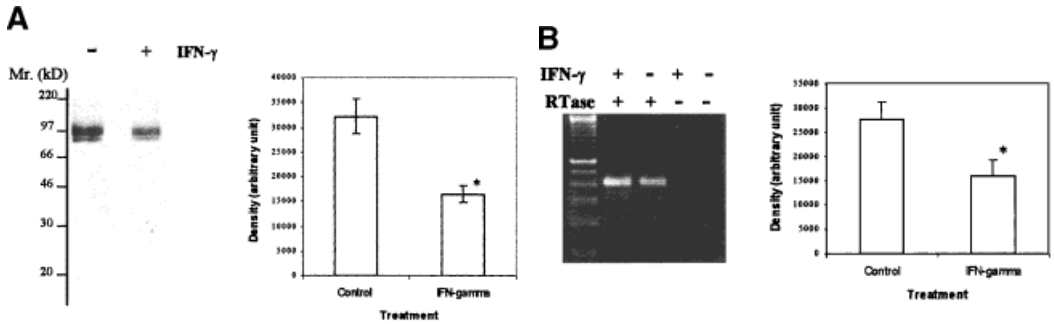

Fig. 3. Downregulation of the IFN-γ receptor. Osteoclasts were cultured for 10 days. After removing non-osteoclast cells, remaining osteoclasts were incubated with IFN-γ (2 ng/ml) for 2 days. Then osteoclasts were lysed and used for Western blot. Alternatively, individual osteoclast was isolated by micromanipulation and subjected to RNA isolation. **A**: Fifty micrograms of protein were subjected to a SDS–PAGE. The presence of the IFN-γ receptor was determined by Western blot analysis. The result shown is one of three experiments performed separately; $*P < 0.01$. **B**: RT-PCR was performed using specific primers of the IFN-γ receptor. Note a reduced amount of IFN-γ receptor mRNA is present in samples stimulated by IFN-γ. The result shown is one of three experiments performed separately; $*P < 0.01$.

were determined. As shown in Figure 5, a 2.3-fold increase of p47 was observed in samples stimulated with IFN-γ. A similar result was also obtained using quantitative PCR to determine the mRNA level of the p91 subunit of the NADPH oxidase. Figure 6 indicates that IFN-γ induced p91 mRNA levels from 8.44 to 14.6 pM in osteoclasts. These data suggest that IFN-γ upregulates the expression of NADPH oxidase, which explains the enhanced generation of superoxide by osteoclasts.

Effect of Antisense Oligonucleotides on Osteoclastic Superoxide Generation

Using antisense oligonucleotides for p47 subunit of NADPH oxidase, not only was IFN-γ-induced superoxide production diminished but also the basal level of osteoclastic superoxide generation greatly declined (Fig. 7). Treatment with antisense oligonucleotides did not affect osteoclast viability (data not shown). This confirms that reduced superoxide production by antisense oligonucleotides did not result from dead osteoclasts. Blocking synthesis of NADPH oxidase by antisense oligonucleotides results in an inhibition of osteoclastic superoxide generation, indicating that NADPH oxidase contributes to osteoclastic superoxide generation and that upregulation of NADPH oxidase by IFN-γ accounts for the increased superoxide production in osteoclasts.

DISCUSSION

In this study, we have demonstrated the presence of the IFN-γ receptor on osteoclasts. Normal function of the IFN-γ receptor requires not only expression but also binding activity.

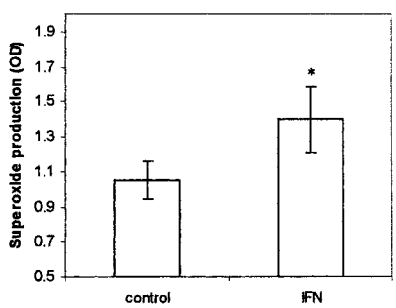

Fig. 4. Effect of IFN-γ on superoxide generation. Osteoclasts were cultured in the chamber slide for 10 days. After removing non-osteoclast cells, the remaining osteoclasts were incubated with IFN-γ (2 ng/ml) for 2 days. Superoxide generation by osteoclasts was determined by NBT reduction assay. Twenty osteoclasts were randomly selected from each experiment and their intensities of NBT staining were quantified by microdensitometry; $*P < 0.01$.

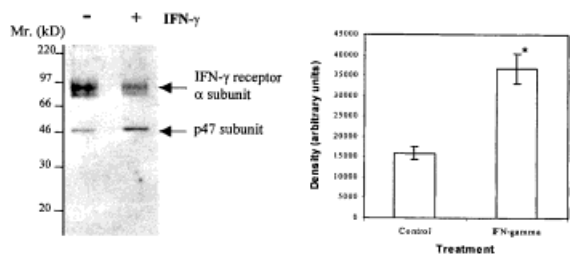

Fig. 5. Effect of IFN-γ on p47 subunit of NADPH oxidase. Osteoclasts were cultured for 1–2 weeks. After removing non-osteoclast cells, remaining osteoclasts were incubated with IFN-γ (2 ng/ml) for 2 days. Then osteoclasts were lysed and 50 μg of protein was subjected to a SDS–PAGE. The same membrane used for determining IFN-γ receptor downregulation (Fig. 3A) was reblotted with p47 antibody. A similar result was observed by two experiments performed separately; $*P < 0.01$.

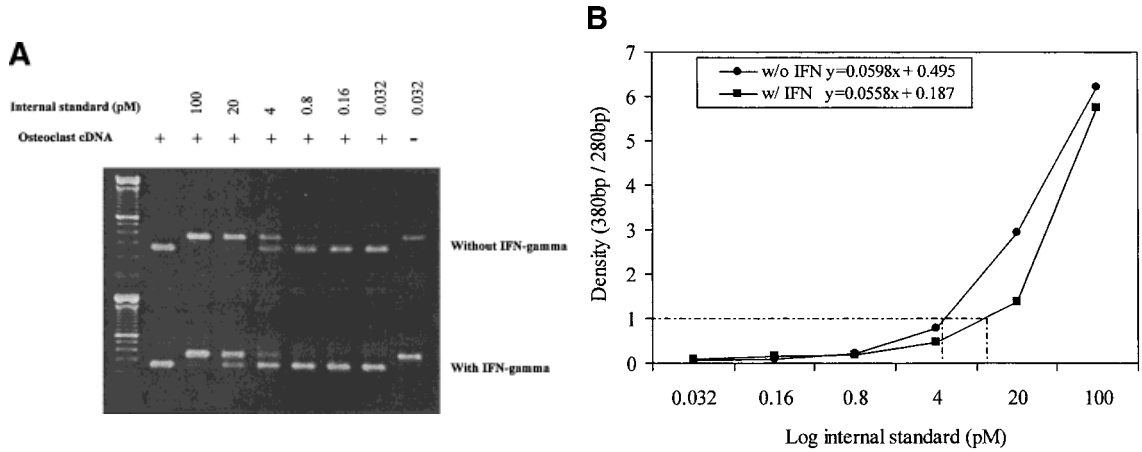

Fig. 6. Demonstration of the increased mRNA level of p91 by quantitative PCR. Osteoclasts were cultured for 2 weeks. After removing non-osteoclast cells by collagenase/dispase (1 mg/ml) digestion and vigorous washings, remaining osteoclasts were incubated with IFN-γ (2 ng/ml) for 2 days. Then total RNA was isolated from osteoclasts and cDNA was obtained using oligo-dT primer. Serial dilutions of the internal standard of p91 were added to each tube containing 5 μl of cDNA. PCR was performed as described in Materials and Methods and the result is shown in Figure 6A. A PCR product of 280 bp was generated from native p91 mRNA and a 380-bp fragment was obtained from the internal standard. The ratio of densities (380 bp/280 bp) was plotted against the concentrations of the internal standard (Fig. 6B). The amount of p91 mRNA present in the sample was defined as the concentration of the internal standard where the ratio equals 1 [Gilliland et al., 1990; Vanden-Heuvel et al., 1993].

We have also shown that ^{125}I-labeled IFN-γ can bind directly to osteoclasts. Binding of IFN-γ to the receptor on osteoclasts results in about a 40% increase of superoxide generation. The presence of the receptor on osteoclasts suggests that the effects of IFN-γ on osteoclasts result from direct stimulation of the osteoclastic IFN-γ receptor. Since NADPH oxidase is an enzyme system contributing to superoxide production by osteoclasts, we further investigated the mechanism of regulation of NADPH oxidase in osteoclasts by IFN-γ. The expression level of p47 and p91, two subunits of NADPH oxidase, was increased with IFN-γ stimulation. This indicates that superoxide production stimulated by IFN-γ occurs through an upregulation of enzyme synthesis. A similar induction of NADPH oxidase components has been related to IFN-γ stimulation of superoxide in white blood cells [Amezaga et al., 1992].

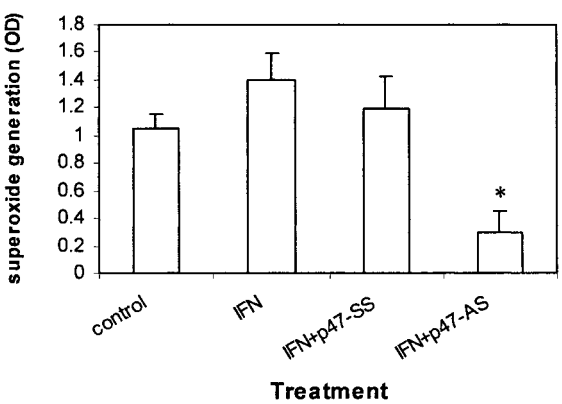

Fig. 7. Effect of antisense oligonucleotides on osteoclastic superoxide production. Osteoclasts were incubated with 2 ng/ml of IFN-γ along with 25 μM of antisense (AS) and the corresponding sense (SS) oligonucleotides for 2 days. No significant difference in osteoclast viability was observed. Then, superoxide generation by osteoclasts was determined with the NBT reduction assay; $^*P < 0.01$.

NADPH oxidase consists of membrane bound p91 and p22, and cytoplasmic subunits p47 and p67. Upon stimulation, p47 and p67 are translocated to the membrane. Assembly of activated NADPH oxidase occurs and results in superoxide generation. In addition, G protein and phosphorylation of cytosol subunits are necessary for activation of NADPH oxidase. In this study, we found a 2.3-fold increase in p47 subunit (Fig. 5) and approximately a 2-fold increase of the p91 subunit, yet superoxide production was only about a 40% increase. Increased levels of enzyme subunits form a foundation to enhance superoxide production, however, it may not reveal an exact 1:1 relationship between the enzyme amount and activity. The enzyme activity can be affected by many factors (post-translational modification, phosphorylation, translocation, and activation process). It has been demonstrated that src tyrosine

kinase affects superoxide generation via a mechanism to challenge the translocation of NADPH oxidase components to the cell membrane, where the functional NADPH oxidase complex is assembled [Yang et al., 2000].

There are many accounts in the literature describing the inhibitory effects of IFN-γ on osteoclastic bone resorption in vitro [Gowen et al., 1986; Hoffmann et al., 1987; Fujii et al., 1990; Roodman, 1993; Lerner, 1996]. The mechanism by which IFN-γ inhibits bone resorption in vitro is controversial. There were reports that IFN-γ blocked IL-1 induced PGE_2 secretion, suggesting that IFN-γ inhibits bone resorption via a PGE_2-dependent pathway [Browning and Ribolini, 1987; Hoffmann et al., 1987]. IFN-γ suppression of osteoclastic collagenase release has been reported [Shen et al., 1988]. It was suggested that inhibition of bone resorption by IFN-γ is a result of nitric oxide synthesis, a strong inhibitor of osteoclast activity [Ralston and Grabowski, 1996; Van't Hof and Ralston, 1997]. Recent studies also observed that recruitment of mature osteoclasts were inhibited by IFN-γ [Fox and Chambers, 2000; Takayanagi et al., 2000]. In this study, we demonstrated the presence of IFN-γ receptors on osteoclasts. This observation provides that the molecular foundation for IFN-γ directly regulates the function and activity of osteoclasts. During bone resorption, osteoclasts generate superoxide [Garrett et al., 1990; Key et al., 1990, 1994]. Thus, additional explanation for inhibitory effect of IFN-γ may be that overproduction of superoxide stimulated by IFN-γ causes damage to osteoclasts which results in decreased osteoclast numbers and reduced bone resorption.

The physiologic role of induced superoxide production by interferon gamma is not completely understood, however, bidirectional regulation of osteoclast function by superoxide could be addressed. First, overproduction of superoxide may also cause cell damage and/or induce apoptosis, thus, osteoclastic bone resorption is reduced. The overproduction of superoxide may be more critical for osteoclastic differentiation and formation. Second, superoxide has been shown to directly contribute to the bone resorption process by fragmentation of bone matrix collagen/proteins [Key et al., 1994]. Increased superoxide generation by interferon gamma may facilitate bone matrix protein degradation, and thus increase osteoclastic bone resorption. This has been observed in the osteopetrotic condition under which superoxide generation is far below the basal level [Reeves et al., 1979; Beard et al., 1986; Yang et al., 1999]. Stimulation with IFN-γ leads to an increase of superoxide from a defective level to an amount that remedies a defect of superoxide generation in osteopetrotic cells.

In conclusion, our study demonstrates the presence of the IFN-γ receptor on osteoclasts. This finding provides evidence that IFN-γ acts directly on osteoclasts. The specific binding of IFN-γ to the osteoclastic receptor results in a stimulation of superoxide generation by osteoclasts.

ACKNOWLEDGMENTS

This work was supported in part by URC in Medical University of South Carolina and General Clinical Research Center at Medical University of South Carolina. We are grateful to Amgen for providing RANKL.

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