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J Dent Res 80(1):351-355, 2001

Interferon-γ Down-regulates Gene Expression of Cathepsin K in Osteoclasts and Inhibits Osteoclast Formation

ABSTRACT

The cytokine, IFN-γ, has been shown *in vitro* to inhibit bone resorption, but the mechanisms responsible for this inhibition have not been clearly defined. Cathepsin K is a major protease responsible for bone resorption. IFN-γ may inhibit bone resorption through down-regulation of osteoclast genes, including cathepsin K. To test the hypothesis, we investigated the effect of IFN-γ on cathepsin K expression in the MOCP-5 and wild-type mouse bone marrow co-culture systems by Northern blot as well as osteoclast formation at different stages of differentiation. The results show that IFN-γ down-regulates mRNA levels of cathepsin K in a time- and dose-dependent manner. Consequently, cathepsin K protein production is also reduced by IFN-γ. Moreover, our results indicate that IFN-γ inhibits osteoclast formation only early in osteoclast differentiation. IL-6 and TNFα did not significantly affect cathepsin K gene expression in osteoclasts. However, IL-1α stimulated gene expression. In conclusion, our data suggest that the actions of IFN-γ on osteoclastic bone resorption may be mediated by its effects on both osteoclast formation at an early stage and osteoclast gene expression in mature osteoclasts.

KEY WORDS: interferon-gamma, cathepsin K, osteoclast formation, gene regulation.

Received December 28, 1999; Last revision October 16, 2000; Accepted October 26, 2000

INTRODUCTION

There has been much interest in cathepsin K since the enzyme was implicated as a major protease responsible for bone resorption. The physiological significance of cathepsin K, highlighted in the recent work of Gelb *et al.* (1996), indicated that mutations in the human cathepsin K gene caused pycnodysostosis characterized by short stature, osteosclerosis, bone fragility, and abnormal bone and tooth development. The abundant and selective expression of cathepsin K in osteoclasts strongly suggests that it plays a specialized role in bone resorption (Li *et al.*, 1995).

Bromme *et al.* (1996) demonstrated that cathepsin K is a highly active cysteine protease capable of hydrolyzing extracellular matrix proteins at pH 5.5. Inui *et al.* (1997) provided further evidence of the crucial role of cathepsin K in osteoclastic bone resorption through their cathepsin K antisense inhibition experiments. Saftig *et al.* (1998), using a knock-out mouse model, confirmed the major importance of cathepsin K in bone remodeling. Their results demonstrated that cathepsin-K-deficient mice display an osteopetrotic phenotype with excessive trabeculation of the bone-marrow space (Saftig *et al.*, 1998). In addition, osteoclasts from these mice manifest a modified ultrastructural appearance and severely impaired resorptive activity (Saftig *et al.*, 1998).

IFN-γ is a multi-functional cytokine that has attracted significant research effort. Its multiple biologic effects include antiviral, antitumor, and cell-growth-inhibitory activity (Ijzermans and Marquet, 1989). IFN-γ is a glycoprotein produced by activated T-lymphocytes and natural killer cells (Trinchieri-G, 1985). Like many other cytokines, IFN-γ also plays a role in bone metabolism (Goldring and Goldring, 1990). In the group of bone-cell modulating factors, IFN-γ is the only cytokine that inhibits bone resorption (Peterlik *et al.*, 1985; Gowen *et al.*, 1986; Bernard, 1997; Hoffmann *et al.*, 1987), but the exact mechanisms responsible for this inhibition are unknown. IFN-γ may have important local effects on osteoclast activity and formation. Previous studies have attempted to clarify the inhibitory mechanisms of IFN-γ by examining its effect on osteoclast formation (Takahashi *et al.*, 1986; Klaushofer *et al.*, 1989). Their results showed that IFN-γ inhibits bone resorption in part by modulating osteoclast formation. However, the precise stage at which this cytokine acts has not been identified.

In this study, we investigated the underlying mechanism of IFN-γ in bone resorption. The results indicate that IFN-γ may inhibit bone resorption through the down-regulation of cathepsin K expression and osteoclast formation.

MATERIALS & METHODS

Cell Culture

The osteoclast precursor cell line, mouse osteoclast precursor cells (MOCP-5), and mouse stromal cell line (MS-12) were used in a co-culture system recently generated in our laboratory and proven to be a useful model in the study of osteoclasts (Chen and Li, 1998). The MOCP-5 were elicited in an osteoclast-inductive co-culture system and immortalized by means of SV40 large T antigen. The co-culture system is unique because MOCP-5 cells are committed osteoclast precursors. MS-12 cells are necessary in this co-culture system as feeder cells to

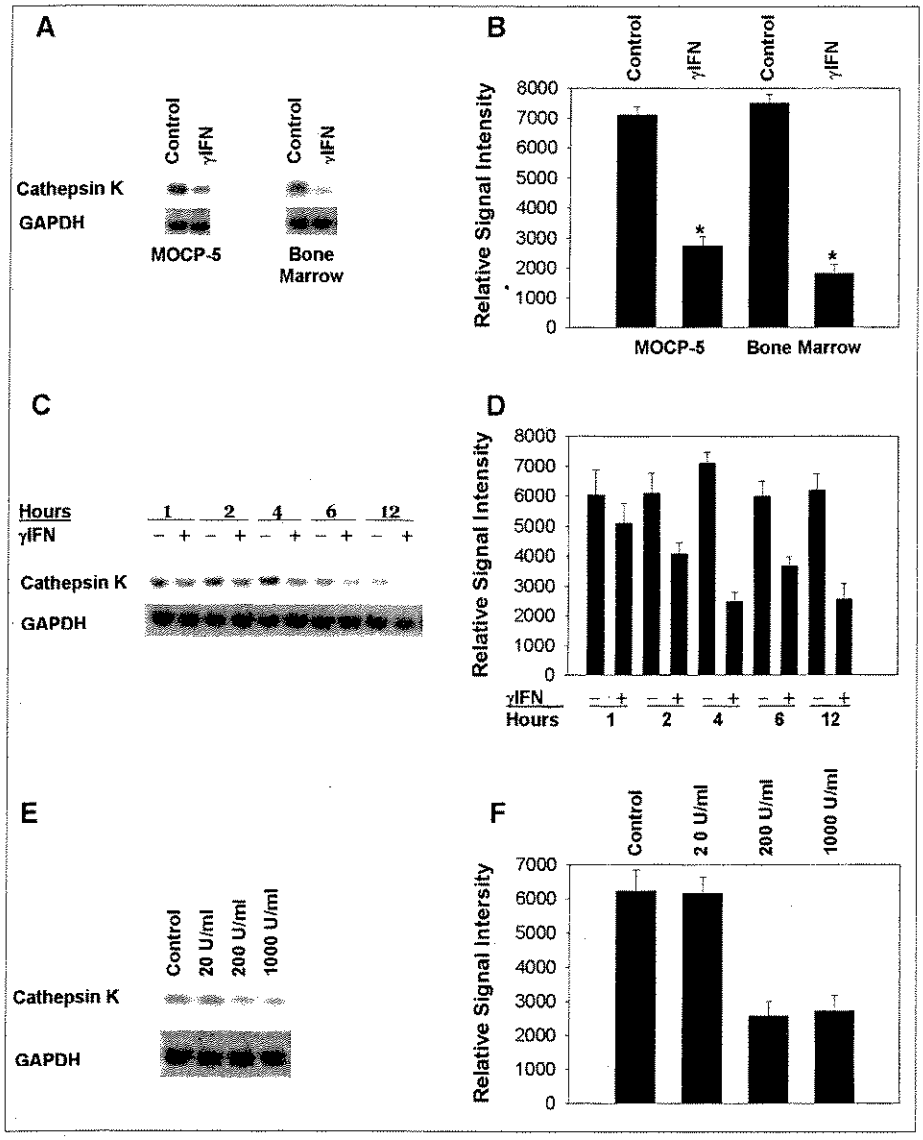

Figure 1. IFN-γ down-regulates the cathepsin K mRNA level in a time- and dose-dependent manner at the transcriptional level. **(A,B)** Down-regulation of the cathepsin K mRNA level by IFN-γ about 2.5-fold in the MOCP-5 co-culture system, and about 3.5-fold in the bone marrow co-culture system when compared with control. **(C,D)** Time response on cathepsin K mRNA expression in the MOCP-5 co-culture system. The decrease in cathepsin K mRNA by IFN-γ was maximal at 4 hrs. **(E,F)** The dose of IFN-γ for maximal decrease effect was 200 U/mL. The quantitative levels of cathepsin K mRNA on Northern blot were evaluated from the value of densitometric scanning and normalized to the level of GAPDH, as shown in the righthand panels. The Figs. represent 4 independent experiments that yielded similar results. * $p < 0.05$; ** $p < 0.01$ compared with control.

support MOCP-5 differentiation into TRAP-positive multinucleated osteoclast-like cells (OCLs).

MOCP-5 (2 x 10^3 cells/well) and MS-12 (1 x 10^5 cells/well) were co-cultured in six-well plates at 37°C for 5 days in a humidified atmosphere of 5% CO_2 in air. Maintenance medium was α-MEM (Life Technology, Gaithersburg, MD, USA) containing 10% FBS, 1 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin in the presence of 10^{-8} M 1,25-dihydroxy vitamin D_3 [1,25-$(OH)_2$ D_3] (Biomol Research Labs, Plymouth Meeting, PA, USA) and 10^{-7} M Dexamethasone (Sigma, St. Louis, MO, USA). 1,25-$(OH)_2$ D_3 and Dexamethasone were not added on day 5, when multinucleated osteoclast-like cells were fully developed, to avoid any influence on the regulation of osteoclast gene expression by IFN-γ. On day 6, mouse IFN-γ (specific activity, 5.08 x 10^6 U/mg; 95% pure, Genzyme Corporation, Cambridge, MA, USA) was added in different doses to each well. After being cultured for various time intervals, the medium was discarded. The multinucleated osteoclast-like cell population was washed in PBS, and total RNA was isolated by means of Trizol reagent (Life Technology, Gaithersburg, MD, USA) as described previously (Chomczynski and Sacchi, 1987). Similar experiments were carried out in a wild-type mouse bone marrow (6 x 10^5 cells/well) and MS-12 (1 x 10^5 cells/well) co-culture system. However, the culture time at which multinucleated osteoclast-like cells were fully developed was 9 days compared with 5 days in the MOCP-5/MS-12 co-culture system. The effects of other cytokines on cathepsin K expression were also evaluated by methods similar to those described above. These cytokines (Genzyme Corporation, Cambridge, MA, USA) included mouse TNF-α (specific activity 5 x 10^7 U/mg; 98% pure), mouse IL-1α (specific activity 1 x 10^7 U/mg; 98% pure), mouse IL-3 (specific activity 1 x 10^7 U/mg; 95% pure), mouse IL-4 (specific activity 1 x 10^7 U/mg; 90% pure), and mouse IL-6 (specific activity 5 x 10^7 U/mg; 95% pure).

Northern Blot Analysis

A 15-μg quantity of total RNA was fractionated by electrophoresis through 1% agarose/formaldehyde gels. The RNA was blotted overnight onto nylon transfer membranes (Boehringer Mannheim Corporation, Indianapolis, IN, USA), pre-soaked in 20 × SSC by capillary action, and fixed by being baked at 80°C for 2 hr. Prior to transfer, each gel was photographed, and the ethidium-bromide-stained bands at 28S and 18S were examined to evaluate the integrity of mRNA and equal loading *per* lane. The nylon membranes were hybridized with radioactively labeled human cathepsin K as a probe. ^{32}P (New England Nuclear, Boston, MA, USA)-labeled radioactive probes were prepared by the random primer labeling procedure according to manufacturer's instructions (Life Technology, Gaithersburg, MD, USA). Hybridizations were performed according to standard protocols (Rosen *et al.*, 1990). After hybridization, the membranes were exposed to autoradiographic films (Kodak XAR-5, Eastman Kodak, Rochester, NY, USA) at -80°C. Analysis of the films was achieved by densitometric scanning (Molecular Dynamics, Sunnyvale, CA, USA). The data were expressed quantitatively by normalization to an internal standard GAPDH.

Cathepsin K Protein Determination

The effect of IFN-γ on cathepsin K protein produced by multinucleated osteoclast-like cells was assessed by ELISA. MOCP-5 (2 x 10^3 cells/well) and MS-12 (1 x 10^5 cells/well) were co-cultured in six-well plates as described above. IFN-γ (200 U/mL) was added on day 6. After 10 or 15 hrs, supernatants were collected and stored at -70°C. Anti-cathepsin K antisera were produced in rabbits as described (Li and Chen, 1999). Rabbit IgG containing anti-cathepsin K was purified by means of ImmunoPure® *Plus* Immobilized Protein, an IgG purification kit (Pierce, Rockford, IL, USA). The purified rabbit IgG containing anti-cathepsin k was coated onto 96-well plates (ICN Biomedicals, Aurora, OH, USA). The test supernatants (100-1000x dilution) was applied and followed by HRP-conjugated anti-human cathepsin K. Colorimetric reactions were developed with *o*-Phenylenediamine (Sigma, St. Louis, MO, USA) in the presence of 0.02% H_2O_2. After 10 minutes' incubation, reactions were stopped with 2 N H_2SO_4 and measured at 490 nm. Purified human recombinant cathepsin K protein was used for preparation of a standard antigen solution. The concentration of cathepsin K in the test solution was interpolated from the standard curve generated from the standard antigen solution.

Osteoclast Formation Assay

MOCP-5 (1 x 10^3 cells/well) and MS-12 (4 x 10^4 cells/well) were co-cultured in 12-well plates under the same conditions as described above. Media containing various concentrations of IFN-γ were added and changed every other day for different time periods. Control media without IFN-γ were also changed every other day. TRAP staining with a commercial kit (Sigma, St. Louis, MO, USA) was carried out when multinucleated osteoclast-like cells matured fully on day 8. An inverted-phase microscope was used to determine the presence and number of multinucleated osteoclast-like cells as described (Chen and Li, 1998). Cells containing three or more nuclei were considered multinucleated osteoclast-like cells. Bone marrow (2.4 x 10^5 cells/well) and MS-12 (1 x 10^6 cells/well) co-culture experiments were carried out in the same manner as the MOCP-5 co-culture system.

Statistical Analysis

Statistical significance was evaluated by Student's *t* test. The results were considered significant for $p < 0.05$.

RESULTS

IFN-γ Down-regulates mRNA Levels of Cathepsin K in a Dose-responsive and Time-dependent Manner at the Transcriptional Level

The mRNA level of cathepsin K was analyzed by Northern blot in the MOCP-5 and bone marrow co-culture system. The results showed that IFN-γ (200 U/mL) down-regulated the cathepsin K mRNA level within 4 hrs in the MOCP-5 co-culture system about 2.5-fold compared with control and in the bone marrow co-culture system about 3.5-fold compared with control (Figs. 1A, 1B). Figs. 1C and 1D show the levels of cathepsin K mRNA in the MOCP-5 co-culture system at different time intervals in the presence of 200 U/mL IFN-γ. The decrease in cathepsin K mRNA was maximal at 4 hrs. The decrease caused by IFN-γ was dose-dependent and with maximal effect when 200 U/mL of IFN-γ was incubated in the co-culture (Figs. 1E, 1F).

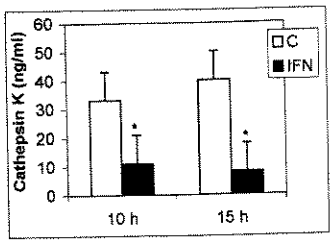

Figure 2. IFN-γ down-regulates cathepsin K protein expression. Three-fold decrease in cathepsin K protein production with IFN-γ for 10 hrs. When the incubation period was extended to 15 hrs, cathepsin K protein decreased about four-fold compared with control. The Figs. represent 3 independent experiments that yielded similar results. *Significant stimulation ($p < 0.05$) compared with control.

IFN-γ Down-regulates Cathepsin K Protein Expression

The effect of IFN-γ on cathepsin K was further evaluated by quantification of cathepsin K protein by the ELISA technique. As shown in Fig. 2, supernatants from multinucleated osteoclast-like cells that were incubated with IFN-γ for 10 hrs showed a three-fold decrease in cathepsin K protein production. When the incubation period was extended to 15 hrs, cathepsin K protein decreased about four-fold compared with control.

IFN-γ Inhibits the Early Stage of Osteoclast Formation in a Dose-responsive and Time-dependent Manner

Recent studies showed that IFN-γ inhibited bone resorption either by inhibiting osteoclast proliferation (Klaushofer *et*

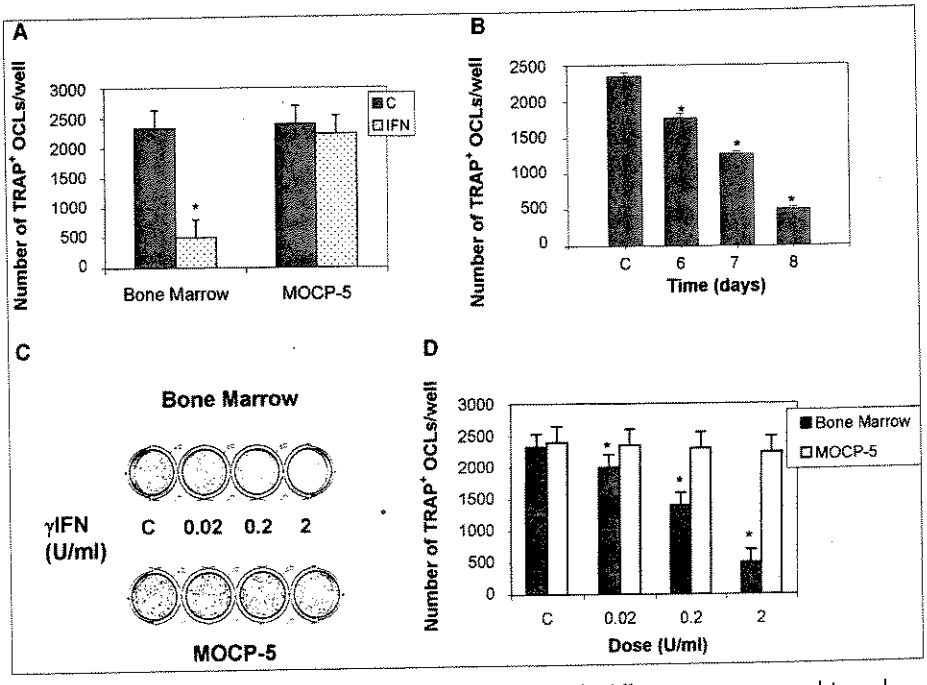

Figure 3. Inhibition of osteoclast formation by IFN-γ at the early differentiation stage and in a dose-responsive and time-dependent manner. **(A)** The number of TRAP-positive osteoclast-like cells (OCLs) formed decreased about 5.5-fold by IFN-γ in the bone marrow co-culture system compared with controls. In this co-culture system, IFN-γ inhibited OCL formation in a time- **(B)** and dose- **(C,D)** dependent manner, whereas in MOCP-5 co-culture, osteoclast formation decreased by only 5% (A). The results are expressed as mean ± SD of 3 independent experiments. * $p < 0.05$ compared with control.

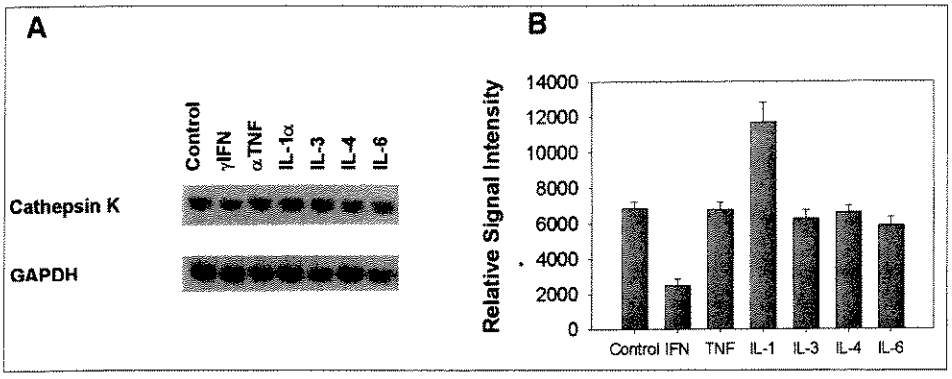

Figure 4. Effects of cytokines on cathepsin K transcription. Up-regulation of cathepsin K gene transcription by IL-1α. No significant effects of other cytokines on cathepsin K transcription. Normalization to GAPDH as shown in (B). The Figs. represent 2 independent experiments that yielded similar results.

al., 1989) or by inhibiting the fusion of osteoclast precursors (Takahashi *et al.*, 1986). To provide details on how IFN-γ affects osteoclast formation and at what differentiation stage, we investigated the effect of IFN-γ on osteoclast using both MOCP-5 and bone marrow co-culture systems. Our recently generated MOCP-5 co-culture system enabled us to investigate the precise stage at which IFN-γ regulates osteoclast formation by comparison with multipotent hematopoietic bone marrow stem cells. Our results demonstrate that the number of osteoclasts formed decreased by 72% when bone marrow co-culture cells were treated with IFN-γ compared with controls (Fig. 3A). In this co-culture system, IFN-γ inhibited OCL formation in a time- (Fig. 3B) and dose- (Figs. 3C, 3D) dependent manner, whereas, in MOCP-5 co-culture, the formation decreased by only 5% (Fig. 3A). These results suggest that IFN-γ prevents bone marrow cells from differentiating into committed osteoclast progenitors.

Effects of Other Cytokines on Cathepsin K Transcription

To examine the expression of cathepsin K response to other cytokines, we incubated TNF-α (500 U/mL), IL-1α (500 U/mL), IL-3 (100 U/mL), IL-4 (30 U/mL), IL-6 (250 U/mL), and IL-8 (500 U/mL) in an MOCP-5 co-culture system on day 6 for 6 hrs. Total RNA extraction and Northern blot analysis were carried out as described above. No significant difference in cathepsin K expression was found between these cytokines and controls, except that IL-1α demonstrated a stimulation effect on gene expression (Fig. 4).

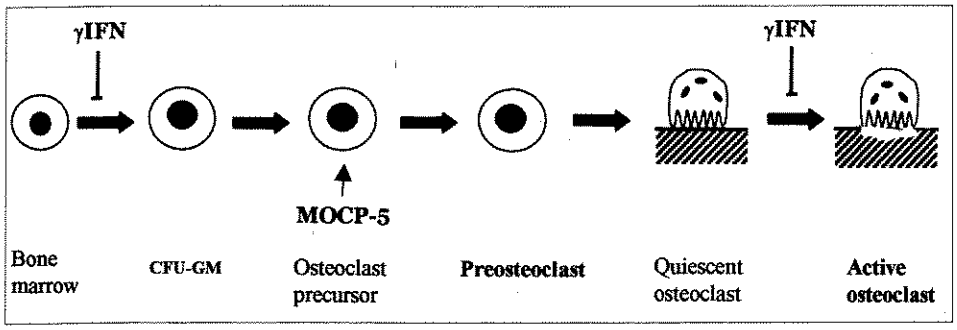

Figure 5. Proposed model for IFN-γ function in osteoclasts. IFN-γ prevents the differentiation of early non-committed osteoclast progenitors and down-regulates osteoclast gene expression.

DISCUSSION

The mechanism underlying IFN-γ inhibition of osteoclast formation and bone has not been fully elucidated. To understand the role of IFN-γ in bone resorption, we examined the effect of IFN-γ on cathepsin K expression. Northern blot analysis showed that cathepsin K mRNA levels are substantially decreased by IFN-γ. Analysis of our data shows that IFN-γ down-regulates the mRNA levels of cathepsin K in a dose-responsive and time-dependent manner. The detailed mechanism of the down-regulation of cathepsin K expression remains to be explored. IFN-γ may activate a transcription repressor to silence cathepsin K mRNA transcription. We have further shown that IFN-γ actually decreased levels of cathepsin K protein. We believe that the reduction of cathepsin K protein production resulted from the reduction of cathepsin K mRNA.

Klaushofer and co-workers (1989) previously reported that IFN-γ inhibits osteoclast proliferation in a time-dependent manner. Takahashi *et al.* (1986) also examined the effects of IFN-γ on the formation of multinucleated osteoclast-like cells in long-term human marrow cultures. The authors suggested that IFN-γ inhibits multinucleated osteoclast-like cell formation by inhibiting fusion of the osteoclast precursors, and that IFN-γ inhibits bone resorption in part by interfering with osteoclast formation. Our recently generated MOCP-5 co-culture system (Chen and Li, 1998) enabled us to investigate the precise stage at which IFN-γ regulates osteoclast formation by comparing committed osteoclast progenitors, MOCP-5, with multipotent stem cells of the hematopoietic bone marrow. In our studies, there was no significant difference in multinucleated osteoclast-like cell formation in the MOCP-5 co-culture system in the presence or absence of IFN-γ, whereas in the bone marrow co-culture system, IFN-γ dose- and time-dependently inhibited the formation of multinucleated osteoclast-like cells. These results suggest that IFN-γ inhibits bone resorption in part by osteoclast formation in the early stage, since MOCP-5 cells are already committed to osteoclast lineage (Chen and Li, 1998). In addition, IFN-γ may inhibit bone resorption in part through cathepsin K expression, as shown in the proposed "IFN-γ in osteoclast action" model (Fig. 5). The more we understand these mechanisms, the better we will know how to use IFN-γ as an immediate or long-term therapeutic agent in bone disorders. Analysis of our data suggests that IFN-γ might be an extremely potent cytokine that can inhibit both osteoclast formation and down-regulate the osteoclastic gene (*i.e.*, cathepsin K), and may play an important role in controlling

bone resorption in inflammatory conditions.

The up-regulation of IL-1α on cathepsin K gene expression is consistent with other studies which demonstrated its powerful osteoclast-activating function, resulting in bone resorption *in vitro* (Gowen and Mundy, 1986) and *in vivo* (Sabatini *et al.*, 1988; Boyce *et al.*, 1989). The underlying regulatory processes for this stimulation are unknown and are being investigated in our laboratory. Although TNF-α and IL-6 were known to stimulate bone resorption, they did not show any effect in cathepsin K expression. TNF-α and IL-6 may regulate bone remodeling through mechanisms that are yet to be characterized. They may be involved in the events of osteoclast formation such as proliferation, differentiation, and fusion of osteoclasts, or regulation of other osteoclast genes.

In conclusion, our results demonstrate that IFN-γ inhibited osteoclast formation and down-regulate cathepsin K expression in a dose-responsive and time-dependent manner. IFN-γ inhibited osteoclast formation at the early stage. The down-regulation of cathepsin K mRNA transcription consequently results in the reduction of cathepsin K protein production. To our knowledge, this is the first demonstration of the regulation by IFN-γ on osteoclasts through osteoclast formation at an early stage of osteoclast differentiation and through cathepsin K expression at a late stage of osteoclast differentiation, as shown in Fig. 5. These results may be of importance in our understanding of the regulatory events involved in the control of bone resorption. This in turn may give more insight into alternative and more efficacious therapeutic interventions in bone disorders such as osteoporosis, osteoarthritis, and periodontal disease.

ACKNOWLEDGMENTS

The authors thank Drs. Christopher V. Hughes and Kalu U. Ogbureke for their critical reading of the manuscript. We thank Wenjie Deng for assistance with manuscript preparation. This work was supported by grants DE-10887 and AR 44741 from the NIH.

REFERENCES

Bernard GW (1997). Osteogenesis and its control. In: Fundamentals of craniofacial growth. Dixon AD, Hoyte DAN, Ronning O, editors. Boca Raton, FL: CRC Press, pp. 45-58.

Boyce BF, Aufdemorte TB, Garrett IR, Yates AJ, Mundy GR (1989). Effects of interleukin-1 on bone turnover in normal mice. *Endocrinology* 125:1142-1150.

Bromme D, Okamoto K, Wang BB, Biroc S (1996). Human cathepsin O2, a matrix protein-degrading cysteine protease expressed in osteoclasts. Functional expression of human cathepsin O2 in *Spodoptera frugiperda* and characterization of the enzyme. *J Biol Chem* 271:2126-2132.

Chen W, Li YP (1998). Generation of mouse osteoclastogenic cell lines immortalized with SV40 large T antigen. *J Bone Miner Res* 13:1112-1123.

Chomczynski P, Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159.

Gelb BD, Shi GP, Chapman HA, Desnick RJ (1996). Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. *Science* 273:1236-1238.

Goldring MB, Goldring SR (1990). Skeletal tissue response to cytokines [review]. *Clin Orthop* 258:245-278.

Gowen M, Mundy GR (1986). Actions of recombinant interleukin 1, interleukin 2, and interferon-gamma on bone resorption in vitro. *J Immunol* 136:2478-2482.

Gowen M, Nedwin GE, Mundy GR (1986). Preferential inhibition of cytokine-stimulated bone resorption by recombinant interferon gamma. *J Bone Miner Res* 1:469-474.

Hoffmann O, Klaushofer K, Gleispach H, Leis HJ, Luger T, Koller K, Peterlik M (1987). Gamma interferon inhibits basal and interleukin 1-induced prostaglandin production and bone resorption in neonatal mouse calvaria. *Biochem Biophys Res Commun* 143:38-43.

Ijzermans JN, Marquet RL (1989). Interferon-gamma: a review. *Immunobiology* 179:456-473.

Inui T, Ishibashi O, Inaoka T, Origane Y, Kumegawa M, Kokubo T, *et al.* (1997). Cathepsin K antisense oligodeoxynucleotide inhibits osteoclastic bone resorption. *J Biol Chem* 272:8109-8112.

Klaushofer K, Horandner H, Hoffmann O, Czerwenka E, Konig U, Koller, *et al.* (1989). Interferon gamma and calcitonin induce differential changes in cellular kinetics and morphology of osteoclasts in cultured neonatal mouse calvaria. *J Bone Miner Res* 4:585-606.

Li YP, Chen W (1999). Characterization of mouse cathepsin K gene, the gene promoter, and the gene expression. *J Bone Miner Res* 14:487-499.

Li YP, Alexander M, Wucherpfennig AL, Yelick P, Chen W, Stashenko P (1995). Cloning and complete coding sequence of a novel human cathepsin expressed in giant cells of osteoclastomas. *J Bone Miner Res* 10:1197-1202.

Peterlik M, Hoffmann O, Swetly P, Klaushofer K, Koller K (1985). Recombinant gamma-interferon inhibits prostaglandin-mediated and parathyroid hormone-induced bone resorption in cultured neonatal mouse calvaria. *FEBS Lett* 185:287-290.

Rosen KM, Lamperti ED, Villa-Komaroff L (1990). Optimizing the Northern blot procedure. *Biotechniques* 8:398-403.

Sabatini M, Boyce B, Aufdemorte T, Bonewald L, Mundy GR (1988). Infusions of recombinant human interleukins 1 alpha and 1 beta cause hypercalcemia in normal mice. *Proc Natl Acad Sci USA* 85:5235-5239.

Saftig P, Hunziker E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W, *et al.* (1998). Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proc Natl Acad Sci USA* 95:13453-13458.

Takahashi N, Mundy GR, Roodman GD (1986). Recombinant human interferon-gamma inhibits formation of human osteoclast-like cells. *J Immunol* 137:3544-3549.

Trinchieri-G PB (1985). Immune interferon: a pleiotropic lymphokine with multiple effects. *Immunol Today* 6:131-136.