BRIEF REPORT

INHIBITION OF INTERLEUKIN-1–INDUCED COLLAGENASE PRODUCTION IN HUMAN ARTICULAR CHONDROCYTES IN VITRO BY RECOMBINANT HUMAN INTERFERON-GAMMA

H. J. ANDREWS, R. A. D. BUNNING, T. A. PLUMPTON, I. M. CLARK, R. G. G. RUSSELL, and T. E. CAWSTON

The production of collagenase by human articular chondrocytes in response to interleukin-1β is inhibited in a dose-dependent manner by interferon-γ (1–1,000 units/ml). The analysis of culture medium samples by Western blotting and the measurement of levels of tissue inhibitor of metalloproteinases suggest that the decrease in measurable collagenase activity is primarily due to the inhibition of procollagenase production. These results provide evidence of a role for interferon-γ in limiting connective tissue degradation.

The cytokine interferon-γ (IFNγ) was originally identified by its antiviral activity, but its major function is probably as an immunomodulator (1,2). A number of recent studies have shown that IFNγ is also able to modulate the metabolism of connective tissue cells. Thus, it has been shown to enhance or induce class II major histocompatibility complex antigen expression, to inhibit collagen synthesis by synovial fibroblasts and chondrocytes (3–6), and to inhibit cytokine-stimulated bone resorption in organ cultures of mouse calvaria (7). These activities suggest that IFNγ may play an important role in modulating the turnover of articular tissue in inflammatory conditions.

We have recently shown that recombinant human IFNγ is able to inhibit interleukin-1 (IL-1)– and tumor necrosis factor (TNF)–stimulated cartilage proteoglycan degradation in vitro and to inhibit IL-1– and TNF-stimulated production of stromelysin (matrix metalloproteinase III) by human articular chondrocytes in monolayer culture (8,9). Since collagen is a major component of the cartilage matrix, we investigated whether IFNγ is also able to inhibit the production of collagenase (matrix metalloproteinase I) by chondrocytes and, in particular, whether it is able to prevent the production of high levels of collagenase in response to IL-1. In addition, we investigated whether the observed decreases in measurable metalloproteinase activity are due to decreased production of the enzyme or whether they could be accounted for by increased production of inhibitors such as tissue inhibitor of metalloproteinases (TIMP).

MATERIALS AND METHODS

Cytokines. Recombinant human IFNγ (2×10^7 units/mg) was a generous gift from Boehringer-Ingelheim (Vienna, Austria) and was >99% pure and endotoxin free. Recombinant human IL-1α and recombinant human IL-1β ($1–2 \times 10^8$ units/mg) were generous gifts from Glaxo Laboratories Ltd. (Greenford, Middlesex, UK).

Chondrocyte culture. Slices of macroscopically normal human articular cartilage were taken from the femoral and tibial condyles of knee joints obtained

From the Rheumatology Research Unit, Addenbrooke's Hospital, Cambridge and the Department of Human Metabolism and Clinical Biochemistry, University of Sheffield Medical School, Sheffield, United Kingdom.

Supported by the British Petroleum Company, the Arthritis and Rheumatism Council, the Nuffield Foundation, and SmithKline Beecham.

H. J. Andrews, PhD: Addenbrooke's Hospital; R. A. D. Bunning, PhD: University of Sheffield Medical School; T. A. Plumpton: Addenbrooke's Hospital; I. M. Clark, BSc: Addenbrooke's Hospital; R. G. G. Russell, PhD, DM, FRCP, FRCPath: University of Sheffield Medical School; T. E. Cawston, PhD: Addenbrooke's Hospital.

Address reprint requests to H. J. Andrews, PhD, Rheumatology Research Unit, Addenbrooke's Hospital, Cambridge CB2 2QQ, United Kingdom.

Submitted for publication January 31, 1990; accepted in revised form June 18, 1990.

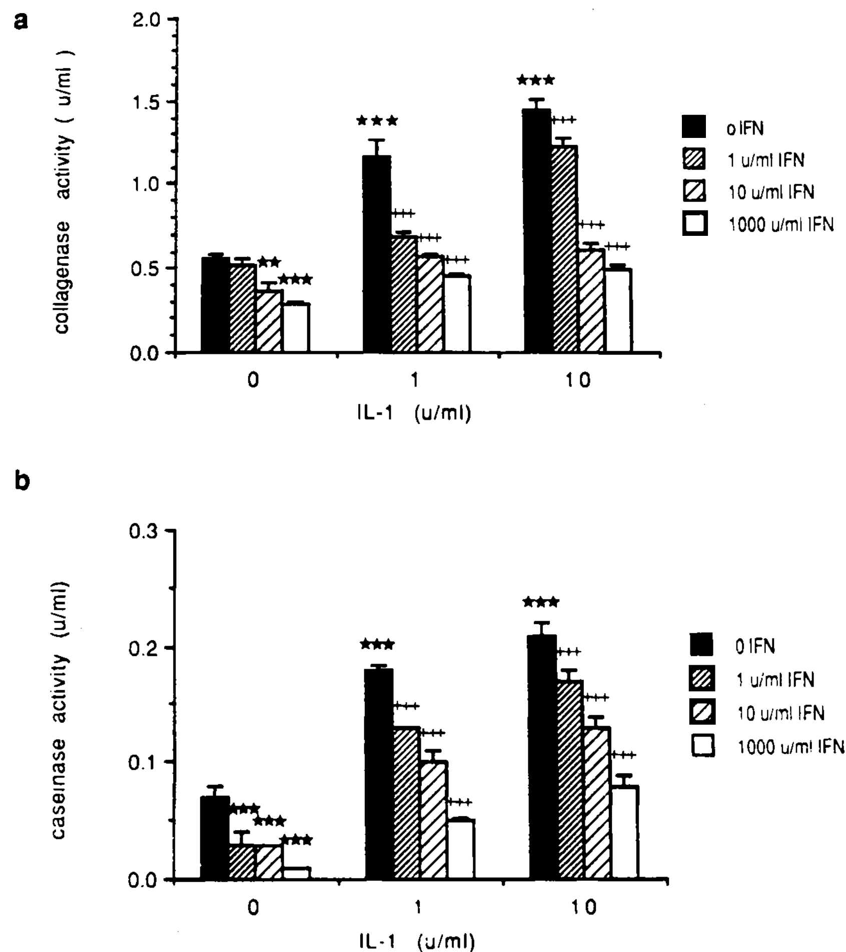

Figure 1. Production of **a,** collagenase and **b,** stromelysin activity by human chondrocytes treated with recombinant human interferon-γ (IFNγ). Human articular chondrocytes were incubated for 48 hours with recombinant human IFNγ in the presence or absence of interleukin-1β (IL-1β; 1 unit/ml or 10 units/ml). The supernatant medium was assayed for collagenase activity following activation with trypsin and APMA, and for stromelysin activity following activation with APMA, as described in Materials and Methods. Results are expressed as the mean and SEM values for 4 replicate wells and are representative of 3 similar experiments. ★★ = $P < 0.01$ and ★★★ = $P < 0.001$ compared with control (0 IL-1), by analysis of variance. +++ = $P < 0.001$ compared with IL-1 (1 unit/ml or 10 units/ml) alone (0 IFN), by analysis of variance.

following amputation for lower limb ischemia and, on 1 occasion, following knee replacement. Cartilage was also taken from a femoral condyle following femoral neck fracture. Chondrocytes were dispersed by sequential enzyme digestion as previously described (10). Monolayer cultures were grown in Eagle's minimal essential medium (EMEM) with Earle's salts (Gibco Europe, Paisley, Scotland) containing 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 m*M* glutamine, and 10% fetal calf serum. Cultures were maintained in a humidified atmosphere of 5% CO_2, 95% air at 37°C. On reaching confluence, cells were passaged into 24-well plates (Falcon, Becton Dickinson, Cowley, UK) at approximately 5×10^4 cells/well, and maintained as above. Serum-containing medium was removed from confluent wells and, after washing, replaced with 1 ml of EMEM containing 1 mg/ml of bovine serum albumin (fraction V; Sigma, Poole, UK) and test substances. Medium was harvested after 48 hours of incubation.

Metalloproteinase assays. Collagenase activity was measured with ^{3}H-acetylated collagen in the dif-

fuse fibril assay (11). Latent enzyme present in the culture medium was activated by preincubation with trypsin (10 μg/ml) for 10 minutes at room temperature, followed by the addition of soybean trypsin inhibitor (50 μg/ml). In addition, APMA (Aldrich, Gillingham, UK) was included in the assay buffer (final concentration 0.67 m*M*). One unit of collagenase digests 1 μg of collagen/minute at 37°C.

Stromelysin activity was measured with ^{3}H-acetylated casein as substrate (12). Latent enzyme was activated by the inclusion of APMA (0.8 m*M*) in the assay buffer. One unit of stromelysin digests 1 μg of casein/minute at 37°C to peptides soluble in 3% (weight/volume) trichloroacetic acid. We have previously shown that the casein-degrading activity produced by human articular chondrocytes in culture is primarily due to a latent metalloproteinase and therefore probably represents stromelysin (8,9). The metalloproteinases are all secreted by connective tissue cells as latent proenzymes that require activation to enable their measurement as described above (13,14).

TIMP assay. TIMP activity was measured in the diffuse fibril assay by its ability to inhibit the activity of a preparation of active collagenase. Latent enzymes in the medium were not activated so that they would not interfere with measurement of TIMP activity. One unit of TIMP inhibits 2 units of collagenase by 50% (13).

DNA assay. The DNA content of culture wells, following incubation with test materials, was determined by the method of Kim et al (15).

Western blotting. Proteins present in culture medium samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (16) and electroblotted onto nitrocellulose paper. This was then incubated with a monospecific rabbit anti-human collagenase IgG, followed by horseradish peroxidase–conjugated pig anti-rabbit IgG antibody (Dako, High Wycombe, UK). Color was developed with 4-chloro-1-naphthol. Prestained molecular weight markers were used (Bio-Rad, Watford, UK).

Controls included active human collagenase from WI38 fibroblasts and collagenase–TIMP complex. Collagenase–TIMP complex was prepared by mixing active human collagenase with excess human TIMP obtained from WI38 fibroblasts.

RESULTS

IFNγ (1–1,000 units/ml) caused a dose-dependent inhibition of the IL-1–stimulated production of procollagenase (Figure 1a). The levels of procollagenase produced in response to 1 unit/ml and 10 units/ml of recombinant IL-1β were significantly inhibited by as little as 1 unit/ml of IFNγ and could be reduced to basal levels by 1,000 units/ml of IFNγ. The production of prostromelysin in response to recombinant IL-1β was inhibited over the same range of concentrations of IFNγ (Figure 1b); this was consistent with our previous report of the effect of IFNγ on chondrocytes treated with partially purified IL-1. Basal levels of collagenase and stromelysin were also decreased by treatment with IFNγ, although higher doses of IFNγ were needed in some experiments, and with chondrocyte cultures from 2 of 5 donors, there was no significant inhibition of basal levels of procollagenase.

IFNγ had a varying effect on the levels of free TIMP. In 2 of 5 cultures, IFNγ alone caused a significant increase in TIMP levels, but this was not consistent, with no stimulation of TIMP being seen in the remaining experiments (Table 1). Results were similarly variable in studies using IFNγ in the presence of IL-1, with a significant stimulation of TIMP in response to IFNγ seen in just 1 of 3 experiments.

The collagenase produced by the chondrocyte cultures was analyzed by Western blotting (Figure 2). Culture medium samples that had not been activated with trypsin and/or APMA showed no detectable active collagenase. All of the collagenase detected had an apparent M_r of ~55K, corresponding to procollagenase (Figure 2, lanes 2–5); this was consistent with previous reports (14). The active collagenase control

Table 1. Results of 2 representative experiments showing tissue inhibitor of metalloproteinase (TIMP) activity produced by human chondrocytes treated with recombinant human interferon-γ (IFNγ)*

IFNγ (units/ml)	TIMP (units/ml)	
	Experiment 1	Experiment 2
0	0.40 ± 0.04	1.43 ± 0.03
0.1	0.34 ± 0.03	1.45 ± 0.06
1.0	0.35 ± 0.06	1.48 ± 0.02
5	0.36 ± 0.03	1.38 ± 0.03
10	0.39 ± 0.09	1.36 ± 0.04
50	0.45 ± 0.05	1.43 ± 0.02
100	0.45 ± 0.06	1.40 ± 0.04
500	0.58 ± 0.05†	1.42 ± 0.01
1,000	0.59 ± 0.05†	1.44 ± 0.04

* Results are the mean ± SEM values for 4 replicate wells. Human articular chondrocytes were incubated for 48 hours with recombinant human IFNγ at the concentrations indicated. The supernatant medium was assayed for free TIMP activity as described in Materials and Methods.

† $P < 0.01$ versus 0 IFNγ, by analysis of variance.

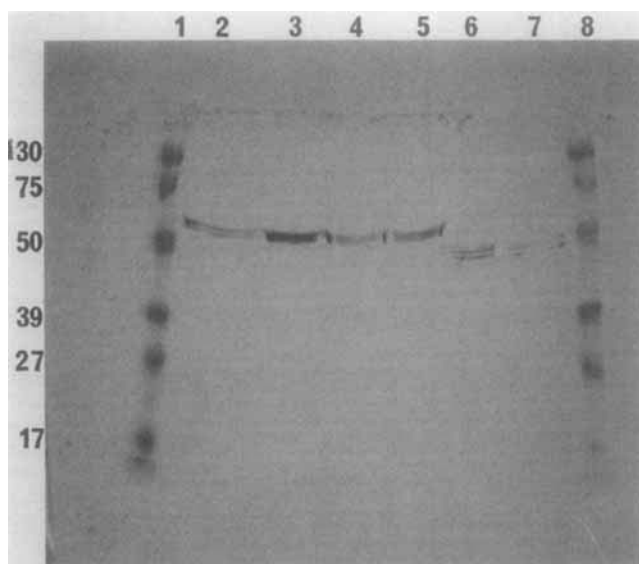

Figure 2. Western blotting analysis of collagenase produced by chondrocytes treated with interferon-γ (IFNγ). Lanes 2–5, Culture medium (40 μl) from chondrocytes treated with IFNγ + interleukin-1 (IL-1) (lane 2), IL-1 alone (lane 3), IFNγ alone (lane 4), or untreated (lane 5). Controls included human collagenase–tissue inhibitor of metalloproteinases complex (lane 6) and active human collagenase (lane 7). Molecular weight markers are shown in lanes 1 and 8. Complex is detected as free active collagenase (M_r ~48K). Only procollagenase is detectable in the chondrocyte media samples (M_r ~55K). Both the active collagenase and procollagenase are detected as doublets, corresponding to glycosylation variants. See Materials and Methods for details.

was detected with an apparent M_r of ~48K (Figure 2, lane 7). TIMP binds only to the active form of collagenase, and the collagenase–TIMP complex dissociates on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and therefore was also detected as free active collagenase (Figure 2, lane 6). The failure to detect active collagenase in either the untreated, IL-1–treated, or IFNγ-treated cultures therefore suggests that there was not a significant amount of collagenase–TIMP complex present in the medium.

The blots showed an apparent increase in the levels of procollagenase antigen in medium from IL-1–treated cultures compared with control cultures (Figure 2, lanes 3 and 5), and an apparent decrease in the levels of procollagenase antigen in medium from cultures treated with IL-1 and IFNγ compared with those treated with IL-1 alone (Figure 2, lanes 2 and 3). Equal volumes of medium from treated and control cultures were loaded onto the gels. The levels of procollagenase detected therefore correspond to that produced by equivalent cell numbers, since there was no significant difference in cell numbers between treated and control cultures, as determined by DNA content (Table 2).

DISCUSSION

Recombinant human IFNγ inhibits the production of procollagenase by human articular chondrocytes treated with IL-1. We have previously reported the inhibition of prostromelysin production by IFNγ (8,9), and these results suggest that the production of these 2 metalloproteinases may be coordinately regulated by IFNγ.

The metalloproteinase levels were measured in a bioassay following activation of the latent enzymes. However, if free TIMP is present in the culture medium in addition to the latent enzymes, then a proportion of the enzymes will bind to TIMP after activation, and therefore will not be measurable in the assay.

It is therefore possible that the decreased levels of metalloproteinase activity detectable in the medium from chondrocytes treated with IFNγ may be due to increased production of TIMP in response to IFNγ, leading to increased enzyme–inhibitor complex formation. In order to investigate this possibility, we measured the levels of free TIMP in the culture medium, prior to trypsin/APMA activation of proenzymes, for all the experiments described. IFNγ had no consistent effect on the levels of free TIMP present in the IL-1–treated cultures; there was stimulation of TIMP in response to IFNγ in only 1 of the 3 experiments (not shown), whereas the levels of collagenase and stromelysin activity were consistently and significantly inhibited by IFNγ. The effect of IFNγ on the level of TIMP activity was equally variable in the absence of IL-1.

Table 2. Results of a representative experiment showing the DNA content of cultures following treatment with interferon-γ (INFγ) and interleukin-1 (IL-1)*

Treatment	DNA (μg/well)
None	0.49 ± 0.02
1 unit/ml IFNγ	0.50 ± 0.03
10 units/ml IFNγ	0.49 ± 0.02
1,000 units/ml IFNγ	0.52 ± 0.03
1 unit/ml IL-1	0.44 ± 0.02
1 unit/ml IL-1 + 1 unit/ml IFNγ	0.52 ± 0.05
1 unit/ml IL-1 + 10 unit/ml IFNγ	0.44 ± 0.02
1 unit/ml IL-1 + 1,000 unit/ml IFNγ	0.52 ± 0.05
10 units/ml IL-1	0.48 ± 0.01
10 units/ml IL-1 + 1 unit/ml IFNγ	0.53 ± 0.04
10 units/ml IL-1 + 10 units/ml IFNγ	0.50 ± 0.05
10 units/ml IL-1 + 1,000 units/ml IFNγ	0.52 ± 0.03

* Results are the mean ± SEM values for 4 replicate wells.

A stimulation of TIMP production could have been masked by enzyme–inhibitor complex formation if there were significant levels of activated enzyme present in the culture medium prior to trypsin/APMA treatment. However, Western blotting of the culture medium samples, using antibodies directed against collagenase, demonstrated that there was no detectable collagenase–TIMP complex present. Furthermore, the levels of procollagenase antigen detectable by Western blotting appear to be lower in cultures treated with IL-1 and IFNγ, compared with those treated with IL-1 alone. Together, the results strongly suggest that the decreased levels of collagenase activity detectable in cultures treated with IFNγ are due to an inhibition of procollagenase production.

IFNγ had no effect on cell numbers, as determined by DNA content of cultures, and we have previously shown that IFNγ (up to 1,000 units/ml) has no effect on chondrocyte viability, nor does it inhibit the production of prostaglandin E_2 by chondrocytes treated with IL-1 (8). This suggests that the inhibition of metalloproteinase production by IFNγ is a specific effect and not a toxic effect or general down-regulation of the response of chondrocytes to IL-1.

The production of metalloproteinases in response to IL-1 may play a key role in the degradation of articular cartilage in conditions such as rheumatoid arthritis. Together, stromelysin and collagenase can degrade all the major matrix components of cartilage (14,17). IL-1 has been shown to stimulate cartilage degradation in vitro (18) and in vivo when injected into rabbits (19), and synovial fluid levels of IL-1 are reported to be high in rheumatoid arthritis (20).

The ability of IFNγ to inhibit the production of high levels of both collagenase and stromelysin by chondrocytes treated with IL-1 suggests that IFNγ may have an important role in limiting cartilage matrix degradation. The demonstration that IFNγ inhibits the production of collagenase is particularly important since collagenase is the only enzyme produced by connective tissue cells that is capable of degrading native collagen at neutral pH (21). IFNγ has also been shown to inhibit bone resorption stimulated by IL-1 in mouse calvarial cultures (7), and a recent report suggests that this could be partially due to the inhibition of collagenase production (22).

In contrast to the high levels of IL-1, little or no IFNγ has been detected in the synovial fluid of patients with rheumatoid arthritis (23,24). Furthermore, recent reports have suggested that IFNγ production may be defective in rheumatoid arthritis patients (25). Our results suggest that these patients may lack a natural protective mechanism capable of limiting the degradation of articular tissues.

Acknowledgments. The authors acknowledge Glaxo Laboratories for their gift of recombinant human IL-1, Boehringer-Ingelheim for their gift of recombinant human IFNγ, and S. Smith for preparation of the manuscript.

REFERENCES

1. Friedman RM, Vogel SN: Interferons with special emphasis on the immune system. Adv Immunol 34:97–140, 1983
2. Trinchieri G, Perussia B: Immune interferon: a pleiotropic lymphokine with multiple effects. Immunol Today 6: 131–136, 1985
3. Amento EP, Bhan AK, McCullagh KG, Krane SM: Influences of gamma interferon on synovial fibroblast-like cells: Ia induction and inhibition of collagen synthesis. J Clin Invest 76:837–848, 1985
4. Jahn B, Burmester GR, Schmid H, Weseloh G, Rohwer P, Kalden JR: Changes in cell surface antigen expression on human articular chondrocytes induced by gamma-interferon: induction of Ia antigens. Arthritis Rheum 30:64–74, 1987
5. Stephenson ML, Krane SM, Amento EP, McCroskery PA, Byrne M: Immune interferon inhibits collagen synthesis by rheumatoid synovial cells associated with decreased levels of procollagen mRNAs. FEBS Lett 180:43–50, 1985
6. Goldring MB, Sandell LJ, Stephenson ML, Krane SM: Immune interferon suppresses levels of procollagen mRNA and type II collagen synthesis in cultured human articular and costal chondrocytes. J Biol Chem 261: 9049–9055, 1986
7. Gowen M, Mundy GR: Actions of recombinant interleukin 1, interleukin 2 and interferon on bone resorption in vitro. J Immunol 136:2478–2482, 1986
8. Andrews HJ, Bunning RAD, Dinarello CA, Russell RGG: Modulation of human chondrocyte metabolism by recombinant human interferon gamma: in vitro effects on basal and IL-1-stimulated proteinase production, cartilage degradation and DNA synthesis. Biochim Biophys Acta 1012:128–134, 1989
9. Bunning RAD, Russell RGG: The effect of tumor necrosis factor α and γ-interferon on the resorption of human articular cartilage and on the production of prostaglandin E and of caseinase activity by human articular chondrocytes. Arthritis Rheum 32:780–784, 1989
10. Meats JE, McGuire MB, Russell RGG: Human synovium releases a factor which stimulates chondrocyte production of PGE and plasminogen activator. Nature 286:891–892, 1980
11. Cawston TE, Barrett AJ: A rapid and reproducible assay

for collagenase using [1-^{14}C] acetylated collagen. Anal Biochem 99:340–345, 1979
12. Cawston TE, Galloway WA, Mercer E, Murphy G, Reynolds JJ: Purification of rabbit bone inhibitor of collagenase. Biochem J 195:159–165, 1981
13. Cawston TE, Murphy G, Mercer E, Galloway WA, Hazleman BL, Reynolds JJ: The interaction of purified rabbit bone collagenase with purified rabbit bone metalloproteinase inhibitor. Biochem J 211:313–318, 1983
14. Chin JR, Murphy G, Werb Z: Stromelysin, a connective tissue-degrading metallo-endopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase: biosynthesis, isolation, characterization and substrates. J Biol Chem 260:12367–12376, 1985
15. Kim Y-J, Sah RLY, Doong J-YH, Grodzinsky AJ: Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem 174:168–176, 1988
16. Laemmli UK, Favre M: Maturation of the head of bacteriophage T4. 1. DNA packaging events. J Mol Biol 80:575–599, 1973
17. Woolley DE, Lindberg KA, Glanville RW, Evanson JM: Action of rheumatoid synovial collagenase on cartilage collagen: different susceptibilities of cartilage and tendon collagen to collagenase attack. Eur J Biochem 50:437–444, 1975
18. Saklatvala J, Pilsworth LMC, Sarsfield SJ, Gavrilovic J, Heath JK: Pig catabolin is a form of interleukin 1. Biochem J 224:461–466, 1984
19. Pettipher ER, Higgs GA, Henderson B: Interleukin 1 induces leukocyte infiltration and cartilage proteoglycan degradation in the synovial joint. Proc Natl Acad Sci USA 83:8749–8753, 1986
20. Duff GW, Dickens E, Wood N, Manson J, Symons J, Poole S, di Giovine F: Immunoassay, bioassay and in situ hybridization of monokines in human arthritis. Prog Leuk Biol 8:387–392, 1988
21. Harris ED, Cartwright EC: Mammalian collagenases, Proteinases in Mammalian Cells and Tissues. Edited by AJ Barrett. Amsterdam, Elsevier/North-Holland, 1977
22. Shen V, Kohler G, Jeffrey JJ, Peck WA: Bone resorbing agents promote and interferon inhibits bone cell collagenase production. J Bone Min Res 3:657–666, 1988
23. Ridley MG, Panayi GS, Nicholas NS, Murphy J: Mechanisms of macrophage activation in rheumatoid arthritis: the role of gamma-interferon. Clin Exp Immunol 63:587–592, 1986
24. Firestein GS, Zvaifler NJ: Peripheral blood and synovial fluid monocyte activation in inflammatory arthritis. II. Low levels of synovial fluid and synovial tissue interferon suggest that γ-interferon is not the primary macrophage activating factor. Arthritis Rheum 30:864–871, 1987
25. Hasler F, Dayer J-M: Diminished IL-2-induced gamma-interferon production by unstimulated peripheral blood lymphocytes in rheumatoid arthritis. Br J Rheumatol 27:15–20, 1988