Transcriptional modulation of cartilage-specific collagen gene expression by interferon γ and tumour necrosis factor α in cultured human chondrocytes

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To examine the possibility that cytokines produced in inflamed joint tissues may contribute to the loss of articular cartilage by causing inhibition of synthesis of cartilage-specific matrix macromolecules, we studied the effects of interferon γ (IFN γ) and tumour necrosis factor α (TNF α), alone and in combination, on the expression of the genes for types-II, -IX and -XI collagens in cultured human chondrocytes. Chondrocytes isolated from human fetal epiphyseal cartilage by sequential enzymic digestions were cultured in the presence of IFN γ (30 pM), TNF α (15 pM) or a combination of suboptimal concentrations of both cytokines (1.5 pM IFN γ plus 0.3 pM TNF α). IFN γ caused a maximal decrease of 23.3–32.6% in the biosynthesis of collagen by chondrocytes. TNF α was a more potent inhibitor causing a 42.8–45.3% decrease at one-half the concentration of IFN γ . A synergistic inhibitory effect of 58.2% was observed with the

combination of 1.5 pM IFN γ plus 0.3 pM TNF α . Electrophoretic analysis of the biosynthesized proteins showed a coordinate decrease in the production of the three cartilage-specific collagen types II, IX and XI. These effects were accompanied by parallel changes in the steady-state levels of their corresponding mRNAs. *In vitro* transcription assays showed that the collagen inhibitory effects of the cytokines occurred largely at the transcriptional level. Similar effects of the cytokines were observed on biosynthesis of types-II, -IX and -XI collagens and steady-state mRNA levels for type-II collagen by chondrocytes obtained from adult articular cartilage. These observations indicate that IFN γ and TNF α can induce a synergistic inhibition of the synthesis of cartilage-specific collagens by fetal and adult human chondrocytes and suggest that these effects may contribute to the articular cartilage loss that occurs in inflammatory joint diseases.

INTRODUCTION

Cytokines are important soluble mediators that participate in cell-to-cell communications in a variety of physiological and pathophysiological processes. It is generally accepted that certain cytokines play an important role in the cartilage damage that occurs in inflammatory joint diseases where removal of extracellular matrix is a prominent feature [1,2]. Several cytokines have been demonstrated in synovium, articular cartilage and synovial fluids from patients with inflammatory arthritis such as rheumatoid arthritis [3–8]. These cytokines initiate a cascade of events that results in the increased production and release of collagenase, gelatinase, stromelysin and other related metalloproteinases [1,2,9–14]. Therefore the damage to articular cartilage that occurs in inflammatory joint diseases has been ascribed to increased degradation of extracellular matrix components by metalloproteinases released by inflammatory cells, proliferative synovium and chondrocytes [15–17]. Although cartilage matrix degradation in these diseases has been well established, the possibility that inhibition of chondrocyte synthesis of cartilage matrix components may play a role in inflammatory joint diseases has not been explored in detail.

The interferons represent a group of cytokines that, in addition to their potent immunomodulatory effects [18], are known to inhibit collagen biosynthesis by dermal [19] and synovial [20,21] fibroblasts and by articular and costal cartilage chondrocytes [22]. It has also been shown that interferons cause a decrease in the steady-state levels of cartilage-specific type-II procollagen mRNA in costal chondrocytes [22]. Low levels of interferon γ (IFN γ) have been detected in synovial fluids and in culture medium from synovial tissue from patients with rheumatoid arthritis [3,6,23]. Tumour necrosis factor α (TNF α), a pro-

inflammatory product of mononuclear cells (reviewed in ref. [24]), is also present in synovial fluids from patients with rheumatoid arthritis [7,8,25]. This cytokine has been shown to reduce markedly the synthesis of cartilage-specific types-II and -XI collagens in rabbit articular chondrocytes [14]. In addition, it has been demonstrated that TNF α stimulates prostaglandin E₂ and collagenase production by synovial fibroblasts and chondrocytes *in vitro* [11–13,26].

Potent synergism between TNF α and IFN γ has been demonstrated regarding their antiviral and antiproliferative effects [27,28], the induction of DR antigen expression in colocal carcinoma cells [29] and pancreatic islet cells [30], and the activation of polymorphonuclear neutrophil functions [31]. These observations prompted us to investigate whether similar synergism occurs in their effects on the biosynthesis of cartilage-specific collagens by fetal and adult human articular cartilage chondrocytes.

MATERIALS AND METHODS

Isolation of chondrocytes

Human fetal epiphyseal cartilage was removed under sterile conditions from femoral heads, knee condyles and tibial plateaus from spontaneous 20–24-week abortions. Normal adult articular cartilage was obtained from 60- and 65-year-old organ transplant donors deceased in motor vehicle accidents. The tissues were obtained from the International Institute for the Advancement of Medicine and Tissue Bank (Lionville, PA, U.S.A.) following protocols reviewed and approved by the National Institutes of Health and in accordance with the U.S.A. and the Pennsylvania Organ Transplant Acts. The tissues were incubated for 1 h at 37 °C in Hank's medium containing 2 mg/ml trypsin and

Abbreviations used: IFN γ , interferon γ ; TNF α , tumour necrosis factor α ; DMEM, Dulbecco's minimal essential medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-phosphate dehydrogenase; 1 $\times$ SSC, 0.15 M NaCl plus 0.015 M sodium citrate.

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2 mg/ml bacterial collagenase, and minced into small pieces. The media were discarded and the minced tissues were digested overnight at 37 °C in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) and 0.5 mg/ml bacterial collagenase. The cells released by the enzymic digestion were filtered through a nylon membrane into a vessel containing fresh DMEM and 10% (v/v) FBS. The cells were collected by centrifugation at 250 *g* for 5 min, resuspended and washed four times with collagenase-free medium. The average yield of fetal chondrocytes was $3(\pm 0.4) \times 10^8$ ($n = 4$) chondrocytes/g wet weight of cartilage and the average yield of adult chondrocytes was 2.6×10^8 chondrocytes/g wet weight of cartilage.

Chondrocyte cultures

The isolated fetal epiphyseal chondrocytes were plated at a cell density of 1×10^7 cells and the adult chondrocytes at a cell density of 1×10^6 cells in 60-mm-diam plastic dishes previously coated with poly(2-hydroxyethyl methacrylate) (PolySciences, Warrington, PA, U.S.A.) according to a procedure modified from that described by Watt and Dudhia [32]. Briefly, the 60-mm-diam dishes were layered with 0.9 ml of a 10% (v/v) solution of poly(2-hydroxyethyl methacrylate) in 95% sterile ethanol, allowed to air-dry overnight under a tissue culture hood, and then exposed to a germicidal u.v. source for 30 min. The culture medium contained DMEM, 10% (v/v) FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, 1% (v/v) vitamin supplements, fungizone and 50 µg/ml ascorbic acid. Previous studies had shown that under these culture conditions the chondrocytes maintained their cartilage-specific phenotype for as long as 240 days (A. M. Reginato and S. A. Jimenez, unpublished work). Two days after plating, the cultures were incubated in the presence of various concentrations of human recombinant IFN γ and human recombinant TNF α (Boehringer-Mannheim Biochemicals, Indianapolis, IN, U.S.A.), alone or in combination, for 24 h. We previously found that exposure of chondrocytes to the cytokines for this period was sufficient to obtain maximum effects and avoided the need to replenish the cultures with fresh cytokines (S. A. Jimenez, A. Diaz and A. M. Reginato, unpublished work).

Biosynthetic studies

One day before labelling, the media were replaced with fresh DMEM containing 10% (v/v) FBS and ascorbic acid (50 µg/ml). On the day of labelling, the media were again replaced with fresh DMEM containing 10% (v/v) FBS, ascorbic acid (50 µg/ml), β -aminopropionitrile (2.5 mM) and the appropriate concentrations of IFN γ and/or TNF α . After 6 h, 5 µCi/ml L-[U-¹⁴C]proline (New England Nuclear, Boston, MA, U.S.A.) or 20 µCi/ml D-[6-³H(n)]glucosamine hydrochloride was added and the cultures were labelled for 18 h. In other experiments, cells were incubated for 6 h with various concentrations of the cytokines alone and in combination and then they were labelled for 16 h with 100 µCi/ml L-[³⁵S]methionine in methionine-deficient culture medium. After labelling, the media were removed and the cell clusters washed with DMEM. The cell clusters were homogenized in a buffer containing 1.0 M NaCl and 50 mM Tris/HCl, pH 7.4, and the following protease inhibitors: 5.0 mM disodium EDTA, 0.2 mM phenylmethanesulphonyl fluoride, 5.0 mM *N*-ethylmaleimide and 1.0 mM *p*-aminobenzamidine hydrochloride. Portions of the cell cluster homogenates were analysed for DNA content by a fluorimetric assay [33]. The media and cell cluster homogenates were extensively dialysed against 0.15 M NaCl plus 50 mM

Tris/HCl, pH 7.4, at 4 °C containing the same concentrations of protease inhibitors. After extensive dialysis, samples were taken for determination of collagen content by a bacterial collagenase assay [34].

SDS/PAGE

The labelled proteins from the media and the cell cluster homogenates were examined by SDS/PAGE in 8% (w/v) polyacrylamide gels under reducing and non-reducing conditions as described previously [35]. In certain experiments, samples of media and cell cluster homogenates were digested with pepsin (100 µg/ml) at 15 °C for 16 h before electrophoresis to convert the type-II procollagens into collagens and to remove the non-collagenous domains of types-IX and -XI collagens. After electrophoresis, the gels were processed for fluorography with ENHANCE (New England Nuclear, Boston, MA, U.S.A.) and exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY, U.S.A.). The collagen composition of the pepsin-digested samples from media and cell cluster extracts was determined and quantified densitometrically using slightly underexposed fluorographs to ensure linearity between the calculated areas and the absolute amount of radioactivity in each band [35]. The relative proportions of types-IX and -XI collagens were calculated as follows: for type-IX collagen, the sum of the values obtained for the bands corresponding to polypeptides of 84 kDa and 72 kDa, which are the expected sizes of migration for the intact type-IX collagen $\alpha 1$ and $\alpha 3$ chains respectively when electrophoresed under reducing conditions [36], was multiplied by the factor 3/2 to obtain the value for the heterotrimer. For determination of the relative proportions of type-XI collagen, the sum of the values obtained from densitometric scanning of the well-separated and distinct pepsin-digested $\alpha 1$ (XI) and $\alpha 2$ (XI) chains was divided by 2 to estimate the amount of the $\alpha 3$ (XI) chain that co-migrated with the $\alpha 1$ (II) collagen chain. The values for the three chains were added to obtain the relative proportion of pepsin-resistant triple-helical type-XI collagen. For type-II collagen the value obtained from the calculations of the $\alpha 3$ (XI) chain was subtracted from the densitometric value of the co-migrating pepsin-digested $\alpha 1$ (II) and $\alpha 3$ (XI) bands. Because the values for all samples were obtained by the same calculations, any error introduced was expected to be a consistent one. The values for the individual collagen types under the various culture conditions were expressed as percentages of the control untreated samples.

Western-blot analysis

Portions of media and cell cluster homogenates were electrophoresed under reducing conditions, and electrotransferred on to nitrocellulose membranes. The membranes were soaked overnight at 4 °C with 5% (w/v) non-fat dry milk in PBS, pH 7.2, and then exposed sequentially to a rabbit polyclonal antibody that recognizes the $\alpha 1$ (II) and $\alpha 3$ (XI) collagen chains (S. A. Jimenez, C. Merryman, L. Ala-Kokko and D. J. Prockop, unpublished work) and a secondary goat anti-(rabbit IgG) monoclonal antibody linked to alkaline phosphatase (Boehringer-Mannheim Biochemicals), and then they were developed using a Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl-1-phosphate solution (Promega, Madison, WI, U.S.A.) as a substrate.

Northern hybridizations

Chondrocytes were cultured in the presence or absence of IFN γ and/or TNF α as described above, and, at the end of the incubation period, the media were removed and the cell clusters from duplicate dishes were washed in Hanks solution and

immediately solubilized in 4 M guanidinium isothiocyanate. Total RNA was isolated in a CsCl discontinuous gradient [37]. For Northern hybridizations, 10 µg samples of total RNA were denatured in formaldehyde, electrophoresed in 0.8 % agarose/formaldehyde gels, and then transferred to nitrocellulose filters. Human cDNAs specific for $\alpha 1(\text{II})$ procollagen [38], type-IX collagen (clone AR 925; A. M. Reginato, C. Baldwin, D. J. Prockop and S. A. Jimenez, unpublished work), type-XI collagen (clone RD 115; R. M. Dharmavaram, C. Baldwin, D. J. Prockop and S. A. Jimenez, unpublished work) and a murine glyceraldehyde-phosphate dehydrogenase (GAPDH) cDNA [39] were nick-translated with [α - ^{32}P]dCTP to a specific radioactivity of $> 10^8$ c.p.m./µg of DNA as described by Rigby et al. [40]. In certain experiments, hybridizations were performed with cDNAs for human collagenase [41] and a DR antigen [42]. The filters were hybridized for 24 h in a 50 % formamide solution and washed under low-stringency conditions. Filters were exposed to X-OMAT AR film (Eastman Kodak) and the relative levels of each transcript determined by laser densitometry of underexposed autoradiographs to ensure a linear correlation between the levels of each transcript and the intensity of the corresponding band.

In vitro nuclear transcription

The transcription rates for various genes were estimated by an *in vitro* nuclear transcription assay [43]. Chondrocytes were incubated for 24 h under control conditions or in the presence of IFN γ , TNF α or a combination of the two. Cells were suspended by trypsinization and nuclei were isolated as described previously [43]. For preparation of hybridization filters, plasmids containing cDNAs for pro $\alpha 1(\text{II})$, $\alpha 1(\text{IX})$ and $\alpha 1(\text{XI})$ collagens, and GAPDH were linearized, denatured and dot-blotted on to nitrocellulose membranes, u.v.-cross-linked and prehybridized at 42 °C in a solution containing 50 % formamide, 5 × Denhardt's solution, 4 × SSC, 0.1 % SDS and 0.1 mg/ml salmon sperm DNA. Nuclear run-off reactions were carried out in the presence of 0.5 mCi [α - ^{32}P]CTP (New England Nuclear; 800 Ci/mmol), 10 mM Tris/HCl, pH 8, 90 mM KCl, 3 mM MgCl $_2$ and 2 mM dithiothreitol for 25 min at 25 °C, and incorporation of [α - ^{32}P]CTP was determined by trichloroacetic acid precipitation of 1 µl portions. The nuclei were digested with proteinase K for 60 min at 42 °C. Samples were extracted with phenol/chloroform, precipitated, resuspended and digested with DNAase. After digestion, the nucleic acids were extracted with phenol/chloroform, precipitated and then resuspended in pre-hybridization buffer. Portions of each sample containing 10×10^6 c.p.m. were adjusted to 400 µl in the same buffer and hybridized to the filters at 42 °C for 48 h. The filters were washed with 0.2 × SSC at 55 °C, digested with RNAase A for 5 min at 37 °C, and dried. Autoradiographs were developed and scanned in a laser densitometer.

RESULTS

Effects of IFN γ and TNF α alone and in combination on collagen synthesis by human fetal epiphyseal chondrocytes

Dose-response experiments showed that IFN γ and TNF α exerted dose-dependent inhibitory effects on chondrocyte collagen synthesis by fetal epiphyseal chondrocytes (Table 1). TNF α was a stronger inhibitor, decreasing collagen biosynthesis by 42.8 % at 15 pM and by 49.5 % at 30 pM, whereas IFN γ caused only a 23.3 % inhibition at 30 pM. To examine whether a combination of the two cytokines exerted a synergistic inhibitory effect on collagen biosynthesis by the fetal chondrocytes, combinations of various concentrations of TNF α plus IFN γ

were examined. The results in Table 2 show that the combination of TNF α and IFN γ exerted a much more pronounced inhibition than either cytokine alone. A maximal inhibition of approx. 70 % was observed with combinations of 1.5 pM TNF α plus 1.5 pM IFN γ . No further inhibition occurred at higher cytokine concentrations. As the combination of 1.5 pM IFN γ plus 0.3 pM TNF α caused nearly maximal inhibition, all subsequent

Table 1 Effects of various concentrations of IFN γ or TNF α on collagen biosynthesis by cultured human fetal epiphyseal chondrocytes

Human fetal epiphyseal chondrocytes were incubated for 24 h with control medium or with media containing increasing concentrations of IFN γ or TNF α and were labelled during the last 18 h of culture with L-[U- ^{14}C]proline as described in the Materials and methods section. Collagen was determined by a collagenase digestion assay [34] in equal portions of triplicate samples from media and cell cluster extracts. The values shown are the means $\pm$ S.D. of triplicate samples.

Culture conditions	Cytokine concentration (pM)	Collagenase-sensitive protein ($10^{-3} \times$ c.p.m./µg of DNA)			Percentage of control
		Media	Cell clusters	Total	
Control		9.4	35.5	44.9 $\pm$ 0.4	—
IFN γ	1.5	8.7	31.4	40.2 $\pm$ 0.6	89.4
	3.0	8.3	30.6	38.9 $\pm$ 0.9	86.6
	15.0	7.8	29.9	37.7 $\pm$ 0.2	83.9
	30.0	7.7	26.7	34.4 $\pm$ 0.9	76.7
TNF α	0.003	9.2	33.0	42.2 $\pm$ 0.8	93.4
	0.03	8.2	30.5	38.7 $\pm$ 1.2	86.2
	0.3	7.9	28.6	36.5 $\pm$ 2.1	81.3
	1.5	7.6	20.6	28.2 $\pm$ 2.5	62.8
	3.0	7.5	19.4	26.9 $\pm$ 3.2	59.9
	15.0	7.5	18.2	25.5 $\pm$ 1.3	57.2
	30.0	6.6	16.1	22.7 $\pm$ 0.2	50.5

Table 2 Effects of IFN γ , TNF α or IFN γ plus TNF α on collagen synthesis by cultured human fetal epiphyseal chondrocytes

Human fetal epiphyseal chondrocytes were incubated for 24 h with control medium or with media containing factors as indicated, and were labelled during the last 18 h of culture with L-[U- ^{14}C]proline as described in the Materials and methods section. Collagen was determined by a collagenase digestion assay [34]. The values shown are means $\pm$ S.D. of three separate experiments each performed with duplicate cultures. Statistical significance of the differences between control and experimental samples was determined by analysis of variance * $P < 0.05$, ** $P < 0.001$.

Culture conditions	Collagenase-sensitive protein ($10^{-3} \times$ c.p.m./µg of DNA)			Percentage of control
	Media	Cell clusters	Total	
Control	11.75 $\pm$ 1.37	36.12 $\pm$ 6.39	47.88 $\pm$ 5.02	—
IFN γ (15 pM)	5.98 $\pm$ 2.43	32.66 $\pm$ 8.99	38.64 $\pm$ 6.56*	80.7
IFN γ (30 pM)	5.92 $\pm$ 2.05	21.30 $\pm$ 2.65	37.22 $\pm$ 0.60*	77.7
TNF α (3 pM)	5.31 $\pm$ 1.60	20.37 $\pm$ 2.72	25.68 $\pm$ 1.12**	53.6
TNF α (15 pM)	5.17 $\pm$ 1.77	18.11 $\pm$ 3.19	23.28 $\pm$ 1.42**	48.6
IFN γ plus TNF α				
0.3 pM + 0.3 pM	4.62 $\pm$ 2.10	20.26 $\pm$ 3.96	24.89 $\pm$ 1.86**	51.9
0.3 pM + 1.5 pM	3.67 $\pm$ 2.17	15.26 $\pm$ 1.95	18.93 $\pm$ 0.22**	39.5
1.5 pM + 0.3 pM	3.34 $\pm$ 2.12	13.88 $\pm$ 1.47	17.22 $\pm$ 0.65**	35.9
1.5 pM + 1.5 pM	2.69 $\pm$ 2.48	12.22 $\pm$ 0.21	14.91 $\pm$ 2.70**	31.1
1.5 pM + 3 pM	3.02 $\pm$ 2.30	13.00 $\pm$ 1.01	16.02 $\pm$ 1.29**	33.4
3 pM + 0.3 pM	2.85 $\pm$ 2.34	13.46 $\pm$ 1.81	16.31 $\pm$ 0.53**	34.0
3 pM + 3 pM	2.71 $\pm$ 2.43	11.60 $\pm$ 0.94	14.31 $\pm$ 1.49**	29.8

Table 3 Effects of IFN γ , TNF α or IFN γ plus TNF α on collagen synthesis by cultured human fetal epiphyseal chondrocytes

Human fetal epiphyseal chondrocytes were incubated for 24 h with control medium or with media containing 30 pM IFN γ , 15 pM TNF α or 1.5 pM IFN γ plus 0.3 pM TNF α and were labelled during the last 18 h of culture with L-[U-¹⁴C]proline as described in the Materials and methods section. Collagen was determined by a collagenase digestion assay. The values shown are the means $\pm$ S.D. of three separate experiments each performed with duplicate cultures. Statistical significance of differences between control and experimental samples was determined by analysis of variance. * $P < 0.05$, ** $P < 0.001$.

Culture conditions	Collagenase-sensitive protein ($10^{-3} \times \text{c.p.m.}/\mu\text{g}$ of DNA)			Percentage of control
	Media	Cell clusters	Total	
Control	10.69 $\pm$ 3.40	36.51 $\pm$ 4.57	47.23 $\pm$ 3.35	—
IFN γ (30 pM)	6.07 $\pm$ 2.04*	25.80 $\pm$ 3.55*	31.84 $\pm$ 5.56**	67.4**
TNF α (15 pM)	6.34 $\pm$ 1.84*	19.49 $\pm$ 4.83**	25.84 $\pm$ 6.26**	54.7**
IFN γ (1.5 pM) plus TNF α (0.3 pM)	4.85 $\pm$ 3.70**	14.92 $\pm$ 8.60**	19.78 $\pm$ 2.18**	41.8**

Table 4 Effects of IFN γ , TNF α or IFN γ plus TNF α on the DNA content, the incorporation of [³⁵S]methionine into total protein and the incorporation of [³H]glucosamine into glycoproteins and glycosaminoglycans by cultured human fetal epiphyseal chondrocytes

Human fetal epiphyseal chondrocytes were incubated for 24 h with control medium or with media containing IFN γ , TNF α or a combination of IFN γ plus TNF α as indicated, and were labelled during the last 18 h of culture with [³⁵S]methionine or [³H]glucosamine as described in the Materials and methods section. The DNA content of the cultures was determined by a fluorimetric assay. The values shown are the means $\pm$ S.D. of three separate experiments each performed with duplicate cultures. Statistical analysis of the differences between control and experimental samples was determined by analysis of variance. No statistically significant differences were found between samples from control and cytokine-treated cultures.

	Total protein ($10^{-3} \times \text{c.p.m.}/\mu\text{g}$ of DNA)	Glycoproteins and glycosaminoglycans ($10^{-3} \times \text{d.p.m.}/\mu\text{g}$ of DNA)	DNA content (μg)
Control	238.19 $\pm$ 5.65	8.23 $\pm$ 0.72	20.18 $\pm$ 1.93
IFN γ (30 pM)	228.15 $\pm$ 11.20	7.89 $\pm$ 0.31	22.33 $\pm$ 2.59
TNF α (15 pM)	229.49 $\pm$ 7.03	8.64 $\pm$ 1.10	19.04 $\pm$ 1.39
IFN γ (1.5 pM) plus TNF α (0.3 pM)	243.83 $\pm$ 11.73	7.97 $\pm$ 0.78	19.85 $\pm$ 4.42

experiments were performed with 30 pM IFN γ or 15 pM TNF α , or with the combination of the two cytokines at suboptimal concentrations (1.5 pM IFN γ plus 0.3 pM TNF α). The results of three separate experiments each performed with duplicate cultures are shown in Table 3. Suboptimal concentrations of both cytokines resulted in a more potent inhibition of collagen biosynthesis than the maximal concentrations of each cytokine alone, indicating that the combination of TNF α and IFN γ exerted a synergistic inhibition of chondrocyte collagen synthesis.

Examination of the relative distribution of collagenase-sensitive proteins between media and cell cluster homogenates indicated that the decrease in newly synthesized collagen caused by IFN γ and TNF α alone or in combination was not due to impaired secretion and subsequent intracellular accumulation of newly synthesized collagen, as a similar level of inhibition was observed in the secreted and cell-cluster-associated collagens. To exclude the possibility that the inhibition of chondrocyte collagen

Figure 1 SDS/PAGE of biosynthesized proteins present in the media and cell clusters of human fetal epiphyseal chondrocytes cultured with various concentrations of IFN γ

The radioactive proteins synthesized by chondrocytes cultured with increasing concentrations of IFN γ were analysed by SDS/PAGE under reducing conditions and fluorography as described in the Materials and methods section. Abbreviation: FN, fibronectin.

Figure 2 SDS/PAGE of biosynthesized proteins present in the media and cell clusters of human fetal epiphyseal chondrocytes cultured with various concentrations of TNF α

The radioactive proteins synthesized by chondrocytes cultured with increasing concentrations of TNF α were analysed by SDS/PAGE under reducing conditions and fluorography as described in the Materials and methods section.

biosynthesis caused by the cytokines (alone or in combination) was due to a global toxic effect, chondrocyte cultures were incubated with the cytokines and labelled with [³⁵S]methionine or [³H]glucosamine. Table 4 shows the effects of the cytokines on [³⁵S]methionine-containing proteins and on [³H]glucosamine-containing glycosaminoglycans and glycoproteins. The results indicate that IFN γ , TNF α or a combination of both at the concentrations used throughout these studies did not substantially affect general protein synthesis or the synthesis of glycosaminoglycans and glycoproteins. Furthermore, the effects of TNF α and IFN γ alone or in combination were not due to cytotoxicity, as chondrocyte viability determined by Trypan Blue dye exclusion (results not shown) and DNA content of cultures exposed for 72 h to various concentrations of TNF α and/or IFN γ were not affected (Table 4).

SDS/PAGE of newly synthesized proteins

Electrophoretic analysis of the proteins synthesized by control cultures and by cultures incubated in the presence of IFN γ alone, TNF α alone or a combination of the two agents (Figures 1–3) did not show any qualitative changes in the patterns of collagens

Figure 3 SDS/PAGE of biosynthesized proteins present in the media and cell clusters of human fetal epiphyseal chondrocytes cultured with IFN γ or TNF α or a combination of suboptimal concentrations of IFN γ plus TNF α

The radioactive proteins synthesized by chondrocytes cultured under control conditions or with IFN γ (30 pM), TNF α (15 pM) or IFN γ (1.5 pM) plus TNF α (0.3 pM) were characterized by SDS/PAGE and fluorography as described in the Materials and methods section. (a) Fluorographs of labelled proteins in the media and cell clusters after electrophoresis under reducing conditions. (b) The same as (a) except that the samples were subjected to limited digestion with pepsin before electrophoresis (denoted by subscript p). (c) Relative proportions of types-II, -IX and -XI collagen synthesized by chondrocytes cultured under the conditions described above obtained from quantitative densitometry from fluorographs shown in (b). ■, Type-II collagen; ▨, type-IX collagen; □, type-XI collagen.

synthesized. Specifically, there was no evidence of a shift to the production of interstitial type-I collagen, as α 2(I) chains were not detected even when overloaded samples were electrophoresed and the fluorographs overexposed. The major protein synthesized in all cultures was type-II collagen which was present as intact or partially processed procollagen chains or as fully processed α chains. Substantial amounts of chains corresponding to types IX and XI collagens were also observed. Treatment of the cultures with either IFN γ (Figure 1) or TNF α (Figure 2) resulted in a dose-dependent reduction in the three cartilage-specific collagen

Figure 4 Western immunoblot of type-II collagen present in the media and cell clusters of human fetal epiphyseal chondrocytes cultured with IFN γ , TNF α or a combination of suboptimal concentrations of IFN γ plus TNF α

Samples of media and cell cluster homogenates from cultures incubated under control conditions or in the presence of IFN γ (30 pM), TNF α (15 pM) or a combination of IFN γ (1.5 pM) plus TNF α (0.3 pM) were examined by Western immunoblot with antibodies that recognize the C-terminal telopeptide of human type-II collagen as described in the Materials and methods section. The antibodies also recognize epitopes on the α 3(XI) collagen chain.

types. The combination of IFN γ and TNF α (Figures 3a and 3b) showed a more pronounced inhibition. Quantitative densitometry of the collagens present in the cell cluster homogenates (Figure 3c) showed that the highest concentrations of IFN γ employed (30 pM) caused a 37% inhibition in the amount of type-II collagen synthesized, whereas the highest concentration of TNF α used (15 pM) caused a 69% inhibition. The combination of suboptimal concentrations of TNF α (0.3 pM) and IFN γ (1.5 pM) caused 79% inhibition of type-II collagen biosynthesis. A synergistic inhibitory effect of IFN γ and TNF α on the synthesis of types-IX and -XI collagens was also demonstrated. IFN γ alone (30 pM) caused a 22% inhibition and TNF α alone (15 pM) caused about 80% inhibition of type-XI collagen synthesis, whereas the combination of the two cytokines at the lowest concentrations examined (1.5 pM IFN γ plus 0.3 pM TNF α) caused 90% inhibition (Figure 3c). The synthesis of type-IX collagen was inhibited by 90% by the combination of suboptimal concentrations of IFN γ and TNF α , whereas 30 pM IFN γ caused only a 60% inhibition and 15 pM TNF α , a 70% inhibition (Figure 3c). These results clearly show synergistic inhibitory effects between IFN γ and TNF α on the synthesis of the three cartilage-specific collagens by human epiphyseal chondrocytes.

Western-blot analysis

To exclude the possibility that the observed effects of the cytokines were due to changes in the intracellular pools of labelled proline, the proteins present in the culture media and in the cell cluster homogenates were examined by Western-blot analysis employing anti-(type-II collagen) antibodies. The results shown in Figure 4 demonstrate that IFN γ (30 pM) or TNF α (15 pM) caused a moderate decrease in the net amount of type-II collagen produced in the cultures, whereas the combination of suboptimal concentrations of the two agents (1.5 pM IFN γ plus 0.3 pM TNF α) was substantially more inhibitory.

Northern hybridizations with cartilage-specific collagen cDNAs

To determine whether the effects of IFN γ and TNF α on cartilage-specific collagen production were reflected at the mRNA level,

Figure 5 Northern hybridization analysis of the steady-state mRNA levels for cartilage-specific $\alpha 1(\text{II})$ procollagen and $\alpha 1(\text{IX})$ and $\alpha 2(\text{XI})$ collagens in human fetal cultured chondrocytes treated with $\text{IFN}\gamma$, $\text{TNF}\alpha$ or a combination of suboptimal concentrations of $\text{IFN}\gamma$ plus $\text{TNF}\alpha$

Total RNA was extracted from human fetal epiphyseal chondrocyte cultures treated with $\text{IFN}\gamma$ (30 pM), $\text{TNF}\alpha$ (15 pM) or a combination of $\text{IFN}\gamma$ (1.5 pM) plus $\text{TNF}\alpha$ (0.3 pM), and 10 μg portions were examined by Northern hybridizations as described in the Materials and methods section. (a) Hybridization with cDNAs for $\alpha 1(\text{II})$ procollagen, $\alpha 1(\text{IX})$ collagen and $\alpha 2(\text{XI})$ collagen. Hybridization of the same filter with a GAPDH cDNA is also shown. (b) Relative proportions of the steady-state mRNA levels for $\alpha 1(\text{II})$ procollagen, $\alpha 1(\text{IX})$ collagen and $\alpha 2(\text{XI})$ collagen determined by quantitative densitometry and corrected by the levels of GAPDH mRNA. ■, Type-II procollagen; ▨, type-IX collagen; □, type-XI collagen.

total RNA from control cultures and from cultures exposed to $\text{IFN}\gamma$ alone, $\text{TNF}\alpha$ alone or a combination of $\text{IFN}\gamma$ and $\text{TNF}\alpha$ at suboptimal concentrations was extracted and examined by Northern hybridizations with human cDNAs specific for types-II, -IX and -XI collagens. The results are shown in Figure 5. To correct for differences in RNA loading, the same filters were hybridized with a control cDNA (GAPDH). Northern hybridizations of the filters with a pro $\alpha 1(\text{II})$ collagen cDNA revealed a pattern of inhibition that corresponded closely to that observed at the protein level. High concentrations of $\text{IFN}\gamma$ (30 pM) or $\text{TNF}\alpha$ (15 pM) caused 50 and 70% decreases respectively in the steady-state mRNA levels for type-II procollagen, whereas the combination of suboptimal concentrations of $\text{IFN}\gamma$ (1.5 pM) plus $\text{TNF}\alpha$ (0.3 pM) caused a decrease of greater than 80%. Similar synergism was observed when the filters were hybridized with cDNAs specific for $\alpha 1(\text{IX})$ and $\alpha 2(\text{XI})$ collagens. In addition, the same filters were hybridized with cDNAs for the DR antigen and for collagenase as positive controls to demonstrate the effectiveness of $\text{IFN}\gamma$ and $\text{TNF}\alpha$ respectively (Figure 6). As expected, $\text{TNF}\alpha$ increased markedly the steady-state levels of mRNA for collagenase, whereas $\text{IFN}\gamma$ induced in the expression of the DR antigen. These results also indicated that the effects of the cytokines on types-II, -IX and -XI collagen mRNA levels were selective for the collagens and not due to a generalized toxic effect.

In vitro nuclear transcription

To determine if the cytokine effects on the steady-state levels of mRNAs corresponding to type-II procollagen and to types-IX and -XI collagens occurred at transcriptional or post-transcriptional levels, *in vitro* transcription assays were performed. The results shown in Figure 7 indicate that $\text{IFN}\gamma$ at

Figure 6 Northern hybridization analysis of the steady-state mRNA levels for human collagenase and DR antigen in cultured human fetal chondrocytes treated with $\text{IFN}\gamma$, $\text{TNF}\alpha$ or a combination of suboptimal concentrations of $\text{IFN}\gamma$ and $\text{TNF}\alpha$

The same filters as shown in Figure 5 were rehybridized as described in the Materials and methods section. (a) Hybridization with cDNAs for collagenase and DR antigen. (b) Relative proportions of the steady-state mRNA levels for collagenase (■) and DR antigen (□) determined by quantitative densitometry and corrected by the levels of GAPDH mRNA.

Figure 7 *In vitro* nuclear transcription of pro $\alpha 1(\text{II})$, $\alpha 1(\text{IX})$ and $\alpha 2(\text{XI})$ collagen genes in fetal human epiphyseal chondrocytes cultured with $\text{IFN}\gamma$, $\text{TNF}\alpha$ or a combination of suboptimal concentrations of $\text{IFN}\gamma$ plus $\text{TNF}\alpha$

Human fetal epiphyseal chondrocytes were incubated for 24 h in DMEM/10% FBS containing either $\text{IFN}\gamma$ (30 pM) or $\text{TNF}\alpha$ (15 pM) or a combination of $\text{IFN}\gamma$ (1.5 pM) plus $\text{TNF}\alpha$ (0.3 pM). Nuclei were isolated and the rates of transcription were determined as described in the Materials and methods section. Labelled transcripts of each sample (10×10^6 c.p.m.) were hybridized to cDNAs for $\alpha 1(\text{II})$ procollagen and for $\alpha 1(\text{IX})$ and $\alpha 2(\text{XI})$ collagens that had been previously dot-blotted and bound to the filter. Hybridizations to GAPDH cDNA and to the plasmid pBR-322 were performed as controls. (a) Autoradiograph of labelled transcripts hybridized to dot-blotted cDNAs. (b) Densitometric analysis of autoradiographs. The integrated areas corresponding to hybridizations to $\alpha 1(\text{II})$ procollagen and $\alpha 1(\text{IX})$ and $\alpha 2(\text{XI})$ collagen cDNAs were normalized with the integrated area corresponding to hybridizations to GAPDH. The relative proportions of the nuclear transcripts were expressed as arbitrary densitometric units. ■, Type-II procollagen; ▨, type-IX collagen; □, type-XI collagen.

Table 5 Effects of IFN γ , TNF α or IFN γ plus TNF α on collagen synthesis by cultured adult human articular chondrocytes

Adult human articular chondrocytes were obtained from normal articular cartilage of three individuals ranging in age from 60 to 65 years and were incubated for 24 h with control medium or with media containing IFN γ , TNF α , or a combination of IFN γ plus TNF α as indicated, and were labelled during the last 18 h of culture with [14 C]proline as described in the Materials and methods section. Newly synthesized collagen was determined by a collagenase digestion assay [34]. The DNA content of the cultures was determined by a fluorimetric assay. The values shown correspond to the means of three experiments each performed with duplicate cultures. Statistical significance of differences between control and experimental samples was determined by analysis of variance. * $P < 0.05$, ** $P < 0.001$.

Culture conditions	Collagenase-sensitive protein ($10^{-3} \times \text{c.p.m.}/\mu\text{g}$ of DNA)			Percentage of control
	Media	Cell clusters	Total	
Control	10.48 $\pm$ 0.35	11.15 $\pm$ 0.53	21.63 $\pm$ 0.44	—
IFN γ (30 pM)	6.48 $\pm$ 0.25*	6.32 $\pm$ 0.46*	12.80 $\pm$ 0.36*	59.2*
TNF α (15 pM)	7.14 $\pm$ 0.68*	4.67 $\pm$ 0.25**	11.81 $\pm$ 0.47**	54.5*
IFN γ (1.5 pM) plus TNF α (0.3 pM)	5.67 $\pm$ 0.58**	3.43 $\pm$ 0.34**	9.10 $\pm$ 0.46**	42.07**

the maximal concentration employed (30 pM) decreased the rate of transcription of the type-II procollagen gene by 40 %, whereas the maximal concentrations of TNF α (15 pM) produced a 60 % inhibition. In contrast, the combination of suboptimal concentrations of the two cytokines caused a very potent inhibition (> 75 %). *In vitro* nuclear transcription assays for the genes encoding types-IX and -XI collagens demonstrated parallel changes, with inhibition by the highest concentrations of the cytokines alone and synergistic inhibitory effects when suboptimal concentrations of the two cytokines were employed.

Effect of IFN γ and TNF α alone and in combination on collagen synthesis by adult articular cartilage

Table 5 shows the effect of IFN γ and TNF α alone and in combination on adult articular cartilage chondrocyte collagen synthesis in three separate experiments each performed in duplicate cultures. Similar inhibitory effects of collagen synthesis were observed in adult articular cartilage chondrocytes to those found in fetal chondrocytes. Suboptimal concentrations of both cytokines (1.5 pM IFN γ plus 0.3 pM TNF α) resulted in a 57.9 % inhibition of collagen synthesis confirming the synergistic inhibition of chondrocyte collagen synthesis observed in fetal chondrocytes.

SDS/PAGE of newly synthesized proteins, Western-blot and Northern hybridization analyses of adult articular cartilage chondrocytes treated with IFN γ , TNF α or IFN γ plus TNF α

Electrophoretic analysis of the proteins synthesized by control cultures of adult chondrocytes, and by cultures incubated in the presence of IFN γ alone, TNF α alone or a combination of the two agents did not show any qualitative changes in the patterns of newly synthesized collagen (Figures 8a and 8b). Similar quantitative changes to those observed in the fetal chondrocytes for type-II and -XI collagen were also observed in the adult articular cartilage chondrocytes. There was no evidence of a shift to the production of interstitial type-I collagen. Quantitative densitometric analysis of newly synthesized type-II collagen

Figure 8 SDS/PAGE of biosynthesized proteins present in the media and the cell clusters of adult articular chondrocytes cultured with IFN γ and TNF α or a combination of suboptimal concentrations of IFN γ and TNF α

Human adult articular cartilage chondrocytes cultured for 24 h in media containing IFN γ (30 pM), TNF α (15 pM) or IFN γ (1.5 pM) plus TNF α (0.3 pM) were labelled with L-[14 C]proline, and portions of media and cell cluster extracts were electrophoresed and the gels processed for fluorography as described in the Materials and methods section. (a) Fluorographs of labelled proteins in the media and cell clusters after electrophoresis under reducing conditions. (b) The same as (a) except that the samples were subjected to limited digestion with pepsin before electrophoresis (denoted by subscript p). (c) Quantitative densitometry of the relative proportions of newly synthesized type-II collagen.

present in the media and cell cluster homogenates (Figure 8c) showed that the highest concentration of IFN γ employed (30 pM) caused a 35 % inhibition in the amount of type-II collagen biosynthesized, whereas the highest concentration of TNF α (15 pM) caused a 60 % inhibition. The combination of suboptimal concentrations of TNF α and IFN γ caused a 65 % inhibition of type-II collagen biosynthesis. As expected, Western-

Figure 9 Western immunoblot of type-II collagen present in the media and cell clusters of adult articular chondrocytes cultured with IFN γ , TNF α or a combination of suboptimal concentrations of IFN γ plus TNF α

Samples of media and cell cluster homogenates from adult articular cartilage chondrocyte cultures incubated under control conditions or in the presence of IFN γ (30 pM), TNF α (15 pM) or a combination of IFN γ (1.5 pM) plus TNF α (0.3 pM) were examined by Western immunoblot with antibodies that recognize the C-terminal telopeptide of human type-II collagen as described in the Materials and methods section. The antibodies also recognize epitopes on the α 3(XI) collagen chain.

Figure 10 Northern hybridization analysis of the steady-state mRNA level for cartilage-specific α 1(II) procollagen in cultured adult articular chondrocytes treated with IFN γ , TNF α or a combination of suboptimal concentrations of IFN γ plus TNF α

Total RNA was extracted from adult articular cartilage chondrocyte cultures incubated in the presence of IFN γ (30 pM), TNF α (15 pM) or a combination of IFN γ (1.5 pM) plus TNF α (0.3 pM), and 8 μ g portions were examined by Northern hybridizations as described in the Materials and methods section. (a) Hybridization with cDNAs for α 1(II) procollagen and GAPDH. (b) Relative proportions of the steady-state mRNA level for α 1(II) procollagen determined by densitometric analysis and corrected for the levels of GAPDH mRNA.

blot analysis employing anti-(type-II collagen) antibodies (Figure 9) confirmed the inhibitory effects of IFN γ (30 pM) or TNF α (15 pM) and the greater inhibitory effect of suboptimal concentrations of the two agents. Northern hybridizations with human type-II collagen-specific cDNA showed that adult articular chondrocytes responded with a more pronounced inhibition by the cytokines than that observed in fetal chondrocytes (Figure 10). High concentrations of IFN γ (30 pM) and TNF α (15 pM) caused a 90–95% decrease in the steady-state mRNA levels for type-II procollagen, whereas the combination of suboptimal concentrations of IFN γ and TNF α almost completely

abolished the expression of the gene as mRNA levels for type-II procollagen were undetectable even when overloaded samples were electrophoresed and the fluorographs overexposed.

DISCUSSION

In inflammatory joint diseases such as rheumatoid arthritis, the inflamed synovial membrane contains polymorphonuclear leucocytes, monocytes/macrophages, lymphocytes and fibroblast-like cells. These various cell types produce and secrete several mediators that modulate the metabolism of chondrocytes in the adjacent cartilaginous tissue. The chondrocytes are not passive bystanders and are actively involved in the destruction of cartilage in these diseases [15–17]. Exposure of chondrocytes to secreted products from inflammatory cells results in profound metabolic changes including their secretion of active and latent forms of collagenase, stromelysin and other related tissue metalloproteinases [9–14]. It is generally accepted that the degradation of cartilage matrix caused by these enzymes plays a crucial role in the loss of cartilage. It has been suggested that a diminished ability of chondrocytes to replace matrix macromolecules may be at least as important, if not the most relevant mechanism responsible for the articular cartilage loss in inflammatory joint diseases [44,45]. The observations presented here that both IFN γ and TNF α inhibit specifically the production of cartilage-specific types-II, -IX and -XI collagens by fetal and adult chondrocytes *in vitro* corroborate this hypothesis. Furthermore, our results clearly showed synergism in the inhibition of chondrocyte cartilage-specific collagen production between IFN γ and TNF α both in embryonic and adult cells. Therefore these results support the notion that cytokine-induced inhibition of chondrocyte matrix production may play a crucial role in pathological conditions associated with articular cartilage damage.

A close correlation was found between the inhibition of collagen synthesis, the steady-state mRNA levels and the *in vitro* nuclear transcription rates for the three cartilage-specific collagens. These observations indicate that inflammation-mediated inhibitory effects of IFN γ and TNF α , alone and in combination, occur largely at the transcriptional level.

Although the results presented here were obtained in an *in vitro* system, the synergism between IFN γ and TNF α on chondrocyte collagen synthesis might have important implications for the modulation of connective tissue metabolism *in vivo*. In fact, Yocum et al. [8] found that IFN γ levels in synovial fluid from patients with rheumatoid arthritis correlated positively with TNF α levels and suggested that IFN γ may indeed influence positively TNF α production. Thus our data suggest that IFN γ may act in concert with TNF α and, perhaps with other cytokines, to contribute to the cartilage destruction observed in rheumatoid arthritis and other inflammatory joint diseases. Similar synergistic effects may also occur between other cytokines. For example, a potent inhibitory effect of interleukin 1 on chondrocyte type-II collagen production has been previously demonstrated [46]. However, synergistic effects of interleukin 1 with TNF α and IFN γ on chondrocyte collagen production have not been studied. A better understanding of the synergistic effects of various cytokines might not only shed light on the pathogenetic events in cartilage damage in inflammatory joint diseases, but may provide new concepts for their therapy.

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