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Immune interferon inhibits collagen synthesis by rheumatoid synovial cells associated with decreased levels of the procollagen mRNAs

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Recombinant immune interferon, (interferon-γ, IFN-γ) inhibits types I and III collagen synthesis by rheumatoid synovial fibroblast-like cells in culture. This decrease is associated with a decrease in the levels of types I and III procollagen mRNAs in these cells as measured by dot blot hybridization. In the control synovial cells the level of α2(I) mRNA is disproportionately high compared with that of α1(I) or α1(III) mRNA, and IFN-γ suppresses the level of α1(I) and α1(III) mRNA to a greater extent than that of α2(I) mRNA. The lymphokine, IFN-γ, may thus have a role in the regulation of collagen synthesis in inflammatory joint disease and other conditions.

Rheumatoid synovial cell *Immune interferon cDNA probe* *Collagen synthesis* *Arthritis* *Procollagen mRNA*

1. INTRODUCTION

In inflammatory joint disease, exemplified by rheumatoid arthritis, a heterogeneous population of cells infiltrates the synovial membrane [1]. Most of the cells in the infiltrate are mononuclear cells, particularly monocyte/macrophages and T lymphocytes. Other abundant cells, however, are fibroblast-like, and are possibly related to normal synovial lining 'B' cells. In primary culture, many of the latter cells have a distinctive stellate appearance and synthesize relatively large amounts of latent collagenase and prostaglandin E_2 (PGE_2) [2]. Cultures of these cells also synthesize and release procollagens, particularly types I and III, as well as fibronectin [3,4]. During the course of studies designed to further characterize these adherent cells from rheumatoid synovium, we observed that some of them expressed HLA-DR (Ia-like) antigens, as assessed by indirect immunoperoxidase techniques with a primary monoclonal antibody directed against HLA-DR [5]. Ia antigens were not detected, however, in cultures of these cells after the first passage, at which time mononuclear cells had largely disappeared. Nevertheless, the adherent fibroblast-like cells continued to synthesize collagens, as shown, for example, by positive staining with a monoclonal antibody specific for human type III collagen. Some monocytes, B lymphocytes and activated T lymphocytes express HLA class II molecules. Immune interferon IFN-γ [6,7] not only augments the expression of class II antigens on these immune cells but also induces the expression of HLA-DR antigens on a variety of nonimmune cells including fibroblasts and endothelial cells [8–10]. When we incubated the synovial fibroblast-like cells with recombinant IFN-γ, we

Abbreviations: IFN-γ, interferon-γ; PGE_2, prostaglandin E_2; cDNA, complementary DNA; SDS–PAGE, SDS-polyacrylamide gel electrophoresis

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also observed the expression of Ia antigens and, in addition, a striking decrease in the extent and intensity of staining with the type III collagen antibody. In vivo, and in primary culture, the IFN-γ would presumably be derived from T lymphocytes in the inflammatory infiltrate.

We show here that IFN-γ decreases the synthesis of types I and III collagens by these synovial fibroblast-like cells, as measured by incorporation of [^{3}H]proline into medium procollagens and, concomitantly, decreases the cellular content of types I and III procollagen mRNAs as assayed by hybridization with cDNA probes of the procollagens α1(I), α2(I), and α1(III).

2. MATERIALS AND METHODS

2.1. *Cell culture*

Synovium was obtained at the time of joint surgery from patients with rheumatoid arthritis. The lining layer was dissected from the underlying capsule and the cells dispersed sequentially with clostridial collagenase and trypsin as described [1,11]. Cultures of the adherent cells were maintained in Dulbecco's modification of Eagle's medium (DME medium, GIBCO) containing 10% fetal calf serum in 10-cm plastic culture dishes (Falcon) in an atmosphere of 5% CO_2 in air at 37°C. After 4–8 weeks in culture and after passage by trypsinization, cytoplasmic RNA was extracted from confluent cells, and in separate cultures collagen synthesis was measured. For comparison, cultured normal human dermal fibroblasts were also used (no.390, provided by Dr R.W. Erbe, Genetics Unit, Massachusetts General Hospital).

2.2. *Collagen synthesis*

Cultures were incubated with or without IFN-γ (prepared by recombinant DNA methodology and kindly provided by Genentech Inc., South San Francisco, CA) in 3.5-cm diameter six-well trays with DME medium, 10% fetal calf serum, for 2 days at 5×10^5 cells/ml. The medium was removed, cells washed 3 times with DME medium, and 1.0 ml fresh DME medium without fetal calf serum containing 50 μg/ml β-aminopropionitrile, 50 μg/ml ascorbic acid, 2 mM glutamine and 20 μCi L-[5-^{3}H]proline (30 Ci/mmol, Amersham Searle, Arlington Hts, IL) was added, and incubation continued for an additional 24 h. The medium was then removed and aliquot portions analyzed by SDS–PAGE (5% acrylamide) [12] with and without reduction with 0.5% β-mercaptoethanol. ^{14}C-labeled rat tail tendon collagen [13] was used as an M_r marker. The labeled collagen in the cell culture medium was further characterized following pepsinization at 4°C, and the collagens analyzed by SDS–PAGE with delayed reduction to distinguish α1(I) from α1(III) chains [14]. Fluorograms of the gels were prepared as described [15].

2.3. *Extraction of RNA*

Cytoplasmic RNA was extracted from confluent synovial cells in the first passage or normal human dermal fibroblasts after several passages (~1×10^6 total cells) after lysing the cells with Nonidet P-40, pelleting the nuclei and treating the cytoplasmic supernate with formaldehyde according to White and Bancroft [16]. Total RNA which served as a human dermal fibroblast standard, was extracted and purified from ~10^8 cells using the guanidine hydrochloride technique [17,18]. RNA solutions were stored at −70°C until analysis. RNA concentration was calculated from the A_{260nm} using the absorbancy index of 21.4 $cm^2 \cdot mg^{-1}$.

2.4. *Preparation of DNA probes*

The cDNA probes used were as follows: human α1(I) procollagen, Hf 677, is of ~1500 bases; human α2(I) procollagen, Hf 1131, is of ~1500 bases; and human α1(III) procollagen, pHC III-I, is of ~1885 bases. Each of these cDNA probes includes a portion of the helical region and the C-terminal propeptide. The cDNA probes for α1(I) [19,20] and α2(I) [20–22] procollagens were kindly provided by Drs F. Ramirez and D.J. Prockop, University of Medicine and Dentistry of the New Jersey-Rutgers Medical School. The cDNA probe for α1(III) procollagen was kindly provided by Drs R.J. Crystal and M. Brantly, National Institutes of Health. In order to compare dot blots, probes had to be similar in size and long enough to be outside of the highly conserved sequences to avoid cross hybridization. Both cloned cDNAs for type I collagen were selected because they were approximately the same size, were highly homologous in terms of their base sequences, and encoded analogous regions of the pro α1(I) and pro α2(I) chains [20]. They did not cross hybridize. The

plasmids, pBR322, containing the cDNA inserts were grown in strains of *Escherichia coli* and isolated from cesium chloride gradients [23]. Each of the probes, 1 μg, was nick-translated using [α-^{32}P]dGTP and [α-^{32}P]dCTP (~700 Ci/mmol, New England Nuclear, Boston, MA) without excision from the plasmid. A nick translation kit was used (BRL, Inc, Gaithersberg, MD). After labeling, the probes were put through two 1-ml Sephadex G-100 columns (Pharmacia P-L Biochemicals, Piscataway, NJ). The specific activities of the labeled probes were as follows: α1(I) procollagen, HF677 = 6.10×10^7 cpm/μg DNA; α2(I) procollagen, HF1131 = 6.36×10^7 cpm/μg DNA and α1(III) procollagen, pHC III-I = 5.20×10^7 cpm/μg DNA.

2.5. *Assay of hybridizable mRNA*

Dot blots were prepared using a filtration apparatus ('Minifold' Schleicher and Schuell, Keene, NH). Appropriate dilutions of the RNA samples were made in 15 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.5) and 100 μl of each were filtered gently onto nitrocellulose paper (B A 85, 0.45 μm pore diameter, Schleicher and Schuell) that had been presoaked in 20 × SSC and dried; 100 μl of 15 × SSC were filtered both before and after addition of the sample. The papers were air-dried and baked for 3 h in vacuo at 80°C. Prehybridization was carried out for 4–6 h at 42°C in a solution containing 50% formamide, 5 × SSC, 0.3% SDS, 0.1% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone and 100 μg/ml sonicated salmon testis DNA, heated at 95°C for 10 min just prior to addition. These conditions were modified from those of Thomas [24]. Total plasmid ^{32}P-labeled probe, 1 μg, along with 200 μg yeast tRNA was taken up in 15 ml of the same solution after being heated at 90°C for 5 min. Hybridization was at 42°C for 24 h. The papers were washed in 0.5 × SSC at 50°C with 4 changes during a 2-h period, then air-dried. Radioautography was performed at −70°C using Kodak X-AR film with DuPont Chronex Lightning-Plus intensifying screen [25]. Quantitation of hybridizable mRNAs was performed by counting the excised dot blots using 'Omnifluor' (New England Nuclear, Boston, MA) or by densitometric analysis of the radioautographs. Scanning densitometry was performed on underexposed autoradiograms of the dot blots where there was a linear response to the concentrations applied. In some cases two exposures of the same gel were scanned to assure the linear response.

3. RESULTS

3.1. *Synthesis of procollagens*

The adherent synovial cells synthesized and released partially processed procollagens as well as a molecule with electrophoretic characteristics consistent with fibronectin (fig.1), as in [3,4]. Fully processed collagen α1(I) and α2(I) chains were not detected in the medium proteins. Incubation of these cells with 100 units of IFN-γ markedly suppressed the synthesis of procollagens (types I and III) and to some extent, fibronectin, as well. This suppression of synthesis of types I and III collagens was also observed after pepsinization of the medium proteins (fig.2). The ratio of α1(I)/α2(I) chains in medium proteins from control cells was ~2.2 as determined by densitometric analysis of the fluorograms. The percent inhibition by IFN-γ of the collagen synthesized and released into the culture medium was 80% for α1(I), 87% for α2(I) and 79% for α1(III). IFN-γ similarly inhibited collagen synthesis by the normal dermal fibroblasts (not shown) in agreement with a recent finding of Jiminez et al. [26].

3.2. *Levels of procollagen mRNAs*

As shown in fig.3, mRNA, which hybridized with the probes for human α1(I) and α2(I) procollagens, was readily detected in cytoplasmic RNA of cultured rheumatoid synovial cells (fig.3A,B) as well as in the cytoplasmic RNA and total RNA extracted by the guanidine-hydrochloride method from the strain of normal dermal fibroblasts (fig.3C,D). The ratio of α1(I)/α2(I) procollagen hybridizable mRNAs in the dermal fibroblast preparations averaged 2.13 for the cytoplasmic RNA and 2.01 for the total RNA (table 1, experiment 1; fig.3) consistent with values previously reported using these probes [20]. In the rheumatoid synovial cell RNA (fig.3A), however, the ratio of α1(I)/α2(I) procollagen hybridizable mRNAs averaged 0.84. The levels of type I procollagen mRNAs in synovial cells

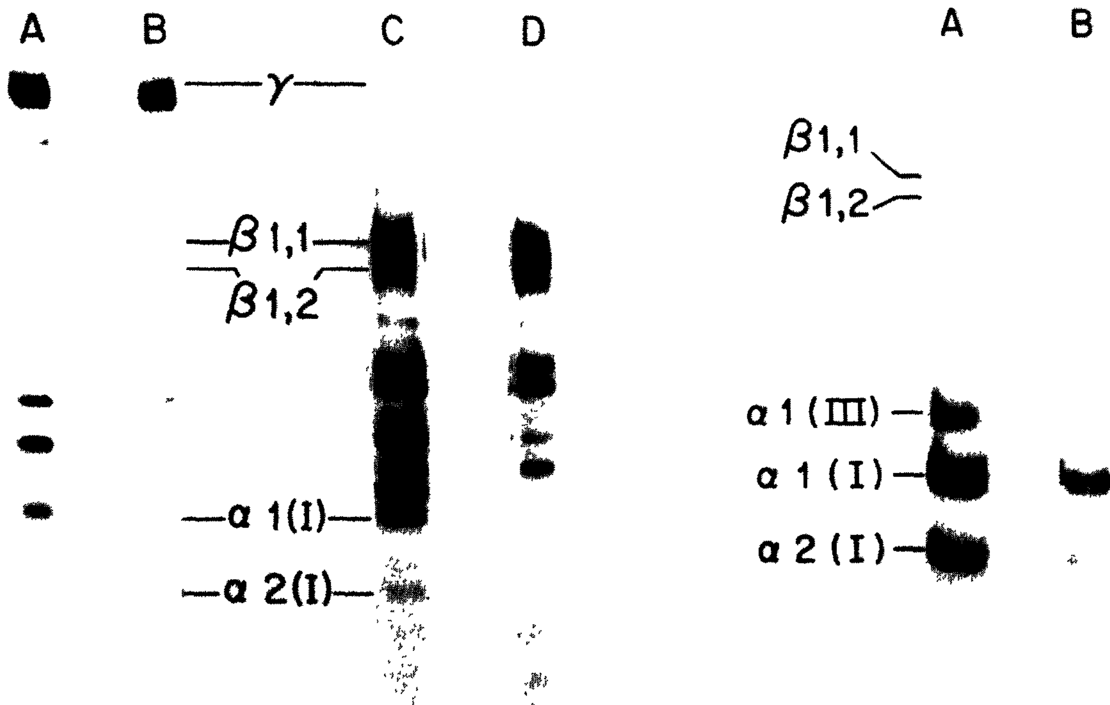

Fig 1. Effect of IFN-γ on proteins secreted by adherent rheumatoid synovial cells Fluorogram of SDS–PAGE of ^{3}H-labeled medium from confluent cells incubated alone (lanes A,C) or with 100 units/ml IFN-γ (lanes B,D) described in section 2. Medium (50 μl) was mixed with 50 μl 2 × sample buffer without (lanes A,B) or with (lanes C,D) 0.5% β-mercaptoethanol. Tubes were stoppered and heated to 60°C for 20 min, then 80 μl aliquots were applied to each lane Film was exposed to gel for 3 days The position of the labeled protein was determined using ^{14}C-rat tail tendon collagen as marker Fibronectin migrates just below collagen γ component under non-reducing conditions (A,B) and in the region of β1,2 under reducing conditions (C,D) The major bands migrating between α-chains and β1,2 components are processed type I and III components.

Fig.2 Effect of IFN-γ on types I and III collagen synthesis by adherent rheumatoid synovial cells. Fluorogram of SDS–PAGE analysis of pepsinized ^{3}H-medium proteins, run using delayed reduction to separate α1(III) from α1(I) chains. Aliquots were from the same media used in fig.1. Medium was from control cells (A) or cells incubated with IFN-γ (100 units/ml) (B) Medium (100 μl) from each was mixed with 100 μl pepsin solution (2 mg/ml pepsin [Worthington] in 1 M acetic acid) at 0°C. After 18 h at 5°C samples were lyophilized to remove the acetic acid; 50 μl H_2O and then 50 μl 2 × sample buffer were added, the tubes stoppered and heated to 60°C for 20 min; 80 μl were applied to each lane with 10% β-mercaptoethanol in 50 μl sample buffer added to wells after samples had run ~2 cm into the separating gel. The film was exposed to the gel for 8 days.

previously incubated with 100 units IFN-γ were markedly depressed (fig.3B). This depression was more striking using the α1(I) procollagen cDNA probe (73% inhibition) compared with the α2(I) procollagen probe (39% inhibition).

Similar results were obtained with a later culture of rheumatoid synovial cells (fig.4; table 1, experiment 2). In control synovial cells (fig.4A), the ratio of α1(I)/α2(I) procollagen mRNAs averaged 1.14. The levels of both these mRNAs were reduced in extracts of cells incubated with 100 units IFN-γ (fig.4B). The pattern of reduction was similar to that observed in fig.3 in that levels of α1(I) procollagen mRNA were suppressed to a greater extent than those of α2(I) procollagen mRNA (77% *vs* 44% inhibition, respectively). It can also be seen

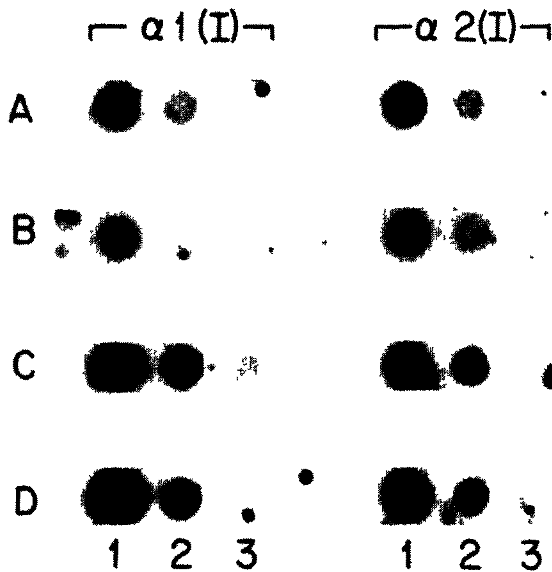

Fig 3. Dot blot hybridization of ^{32}P-labeled α1(I) and α2(I) procollagen probes to cytoplasmic RNA from human rheumatoid synovial cells and cytoplasmic and total RNA from dermal fibroblasts. Cells were passaged 4 days prior to use. Cytoplasmic RNA was extracted from one 10-cm diameter culture dish for each culture condition, which contained ~1 × 10^6 cells. Dilutions were made in 15 × SSC and the cytoplasmic RNA from the equivalent of ~5 × 10^4, 1 × 10^4 and 2 × 10^3 cells applied to the nitrocellulose paper in vertical rows 1, 2 and 3, respectively. (A) Rheumatoid synovial cells; (B) rheumatoid synovial cells incubated with 100 units/ml IFN-γ for 48 h prior to extraction of RNA; (C) human dermal fibroblasts; (D) total RNA from human dermal fibroblasts filtered at ~2.0, 0.40 and 0 08 μg RNA. The same amounts were used for both probes and filtered on the same sheet.

in fig.4 that mRNA for α1(III) procollagen is present in these synovial cells but at a lower abundance than that for the α1(I) or α2(I) procollagens. Ratios of α1(I)/α1(III) procollagen mRNAs were approx.17 in the dermal fibroblasts (fig.4C) and approx.4 in the synovial cells not incubated with IFN-γ (fig.4A). IFN-γ also suppressed the levels of α1(III) procollagen mRNA in the synovial cells (fig.4B) (68% inhibition) consistent with the observed suppression of synthesis of type III collagen present in medium proteins (fig.2).

The IFN-γ also suppressed levels of type I procollagen mRNAs in the dermal fibroblasts. This effect (20% inhibition) was observed at 10 units/ml IFN-γ (not shown).

Table 1

Levels of mRNA for types I and III procollagens in rheumatoid synovial fibroblast-like cells and normal dermal fibroblasts

Experiment	Cell-type source	mRNA			
		Ratio pro α1(I)/pro α2(I)	% Decrease by IFN-γ		
			pro α1(I)	pro α2(I)	pro α1(III)
1	Normal dermal fibroblast	2.13[a]			
		2.01[b]			
	Rheumatoid synovial cell	0.84[a]			
	Rheumatoid synovial cell + IFN-γ	0.38[a]	73	39	
2	Normal dermal fibroblast	2.30[b]			
	Rheumatoid synovial cell	1.14[a]			
	Rheumatoid synovial cell + IFN-γ	0.50[a]	77	44	68

Levels of mRNAs were quantitated densitometrically from the autoradiograms shown in fig.3 (experiment 1) and fig.4 (experiment 2). RNA preparations. [a] cytoplasmic; [b] total

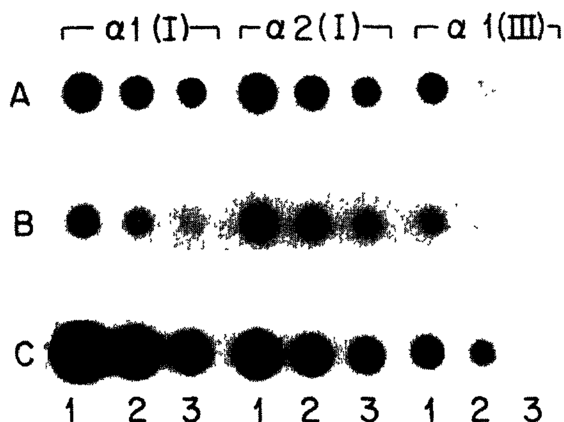

Fig 4. Dot blot hybridization of α1(I), α2(I) and α1(III) procollagen probes to cytoplasmic RNA from human rheumatoid synovial cells and total RNA from human dermal fibroblasts In another experiment (table 1, experiment 2) cells were grown to confluency (~1 × 10^6/10 cm diameter dish) and passaged 28 days prior to extraction of cytoplasmic RNA. Procedures same as in fig.3. The cytoplasmic RNA dilutions were from the equivalent of ~6 × 10^4, 3 × 10^4 and 1.5 × 10^4 cells, respectively (vertical rows 1, 2 and 3), while total RNA from control dermal fibroblasts contained ~1.0, 0.50 and 0.25 μg RNA. (A) Rheumatoid synovial cells; (B) rheumatoid synovial cells with 100 units/ml IFN-γ for 48 h prior to extraction of RNA, (C) total RNA from normal dermal fibroblasts.

4. DISCUSSION

It is evident from the results reported here that IFN-γ inhibited collagen synthesis by the synovial cells, as shown by the decrease in incorporation of labeled proline into medium types I and III procollagens, which was accompanied by strikingly decreased levels of hybridizable types I and III procollagen mRNAs. We do not yet know whether these decreases are accounted for by inhibition of transcription of the procollagen genes, altered processing of the transcript, altered transport from nucleus to cytoplasm or altered stability of the processed transcript [27]. If the decreased levels of these mRNAs do involve a decrease in transcription, then this effect has to be expressed at several different loci, since the human genes for the α1(I) and α2(I) procollagens are located on different chromosomes, 17 and 7, respectively [28,29]. By analogy, in other conditions in which the levels of procollagen mRNAs are suppressed parallel to suppression of collagen protein synthesis, such as infection with Rous sarcoma virus, the effect is probably exerted at the level of transcription [30,31]. On the other hand, Focht and Adams [32] have recently shown that control of type I collagen gene expression may be exerted at both transcriptional and posttranscriptional levels.

Of interest is our finding in the synovial cell preparation examined that the ratio of α1(I)/α2(I) procollagen mRNAs was considerably less than 2, the ratio predicted and observed in previous studies utilizing these cDNA probes [20] and confirmed by us in the normal strain of fibroblasts. The reasons for the disproportionately high levels of the α2(I) procollagen mRNA (or disproportionately low levels of the α1(I) procollagen mRNA) are not yet apparent. The relative increase in the levels of α2(I) procollagen mRNA was not, however, accompanied by an increase in secretion of pro α2(I) chains, since the ratio of medium α1(I)/α2(I) chains was greater than 2. We have not yet obtained sufficient numbers of adherent rheumatoid synovial cells to characterize the hybridizable species and determine whether the hybridizable α2(I) procollagen mRNA can be shown to consist of the normally processed 6.2, 5.7 and 5.5 kb mRNAs [33].

The adherent synovial cells which persist in cultures prepared from the synovial lining of joints from patients with rheumatoid arthritis are fibroblast-like. In addition to collagenase, they synthesize types I and III procollagens and fibronectin but they can be induced to express Ia-like antigens with IFN-γ. In early passages, however, they differ from fibroblasts cultured from dermis in that they produce higher amounts of collagenase and prostaglandins, the levels of which are increased many fold over that of dermal fibroblasts when incubated with the interleukin-1-like molecule, mononuclear cell factor [1]. The decreased ratio of α1(I)/α2(I) procollagen mRNAs, compared to skin fibroblasts may indicate an additional difference among these cells.

Our studies have demonstrated that IFN-γ could be one of the lymphokines that inhibits collagen synthesis in vivo. Mononuclear cells produce both inhibitors and stimulators of collagen synthesis [1,3,4,34–40]. We and others have demonstrated that PGE_2 can inhibit collagen synthesis and secre-

tion in certain cells [3,4,41,42], but it is unlikely that IFN-γ is acting through control of arachidonate metabolism. IFN-γ does not alter medium PGE_2 synthesis induced by mononuclear cell factor [43] IFN-γ is thus a potentially important natural modulator of collagen synthesis in inflammation.

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