Rapid Publication

Selective Inhibition of Human Diploid Fibroblast Collagen Synthesis by Interferons

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Abstract. The effects of α- and γ-interferons (IFNs) on collagen production by confluent human diploid fibroblasts in culture were examined. It was found that partially purified α-IFNs and affinity purified γ-IFNs caused >50% inhibition of collagen synthesis by these cells independently of their effect on cell proliferation. Recombinant α-IFNs showed a similar effect (38.8% inhibition), indicating that collagen synthesis inhibition was a constitutive property of IFNs. Collagen synthesis inhibition by IFNs was concentration dependent. Gel filtration chromatography of the newly synthesized proteins from the media of fibroblasts incubated with partially purified α-IFNs demonstrated a selective depression of molecules eluting in the region of procollagen. No detectable increase in collagen degradation products or underhydroxylation of procollagen was observed. Short-term kinetic studies further demonstrated that the major effect of IFNs was due to a net decrease in fibroblast collagen production rather than to impairment of secretion or increased extracellular degradation of the newly synthesized molecules. These results indicate that α- and γ-IFNs are potent inhibitors of human fibroblast collagen production and suggest that they may play an important role in the regulation of normal and pathologic fibrogenesis.

Introduction

Under normal conditions an accurate balance is maintained between the amount of collagen synthesized by fibroblasts and the amount of collagen removed from the tissues (1). Abnormalities in collagen regulation may be responsible for the excessive and frequently progressive fibrosis characteristic of certain diseases such as scleroderma (2). Collagen production can be controlled by three general mechanisms: modulation of the steady state level of messenger RNA (mRNA); control of mRNA translation; and variation in the fraction of collagen that is degraded intracellularly (1). In addition to these intrinsic mechanisms of control, extrafibroblastic factors may influence the rates of collagen synthesized by these cells. For example, the role of inflammatory cells on the regulation of fibroblast proliferation and collagen synthesis has recently been emphasized (3–8). Interest in the study of this possible mechanism of fibroblast regulation has been stimulated by the frequent observation of a proximity of inflammatory cells and fibroblasts in tissues undergoing active collagen deposition. It has been previously shown that interferons (IFNs)[1] can influence the proliferation of various fibroblastic cell lines (for review see reference 9). The possibility that IFNs may be able to modulate fibroblast collagen synthesis independently of their effects on cell proliferation, however, has not been examined. In the present work, we have studied the effects of various IFN preparations on collagen synthesis, employing confluent dermal fibroblasts to dissociate the antiproliferative effects of IFNs from those related to regulation of collagen synthesis in stationary-phase cells. We found that both α- and γ-IFNs caused a concentration-dependent inhibition of collagen synthesis by normal human diploid fibroblasts. These results suggest that IFN may play an important role in the regulation of collagen production, particularly under pathologic conditions characterized by an accumulation of inflammatory cells in the affected tissues.

Methods

Partially purified α-IFNs (IF-2810), various preparations of α-IFN purified to homogeneity by high pressure liquid chromatography (HPLC) (IL-b, IL-c, IL-d, and IL-null) as described in detail elsewhere

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1. *Abbreviations used in this paper:* aff-IFN-γ, affinity-purified γ-interferon; HPLC, high performance liquid chromatography; IFN, interferon; IFN-A, recombinant α-IFN(A); PMSF, paramethylsulfonyl fluoride.

(10), and a preparation of recombinant α-IFN(A) (IFN-A) were kindly supplied to us by Dr. Sidney Petska (Hoffmann-LaRoche, Inc., Nutley, NJ). All preparations were standardized to contain 1,000 U/ml of antiviral activity (11). A preparation of affinity-purified γ-IFN (aff-IFN-γ) was purchased from Interferon Sciences, Inc. (New Brunswick, NJ) and was also standardized to contain 1,000 U/ml of antiviral activity, based on the information about activity given by the manufacturer.

Fibroblast cultures and labeling conditions. Fibroblasts were obtained from skin biopsies from normal individuals, as described previously (12), and were cultured in modified Eagle's medium supplemented with 10% fetal calf serum in an atmosphere of 5% CO_2 for 5 d to allow the cultures to reach confluence. Previous experiments demonstrated that after confluence, the cultures did not display detectable cell division as measured by serial cell counts, DNA assays, or [^{3}H]thymidine incorporation. The media were removed and 1.5 ml of fresh media containing various concentrations of IFNs, 5% fetal calf serum, and 50 μg/ml ascorbate in modified Eagle's medium were added. After 48 h the media were replaced with the fresh media containing, in addition, from 2 to 4 μCi/ml [^{14}C]proline and 100 μg/ml β-aminoproprionitrile, and the incubation was continued for 24 h. To examine the effects of IFNs on fibroblast proliferation and viability, separate plates were cultured exactly as described above except that the isotopes were omitted. At the end of the experiment the media were aspirated off and the cell layers were detached by trypsinization. The fibroblasts were resuspended in fresh media and their viability was determined by trypan blue exclusion. The number of cells in each plate was then counted by the use of a hemocytometer.

Analyses of labeled proteins. After the 24-h incubation with [^{14}C]proline, the media were removed and the following reagents were added to the following final concentrations: sodium dodecyl sulfate (SDS), 1%; mercaptoethanol, 1%; EDTA, 4 mM; and paramethylsulfonyl fluoride (PMSF), 10 μg/ml. EDTA and PMSF were added to the samples to prevent collagenolytic and proteolytic activity during further processing and analysis. The samples were heated at 100°C for 2 min and then dialyzed against 0.01 M sodium phosphate, pH 7.4, 0.1% mercaptoethanol, and 0.1% SDS. The monolayers containing cells and adherent protein were solubilized in 1% SDS-phosphate buffer containing 4 mM EDTA and 10 μg/ml PMSF, and the solutions were heated and dialyzed as described for the media. The amount of radioactive hydroxyproline synthesized by the cultured fibroblasts was measured by a specific chemical assay (13). In certain experiments both media and cell layers were analyzed, but in most cases only the culture media were studied since, as shown previously, >80% of total collagen synthesized by the control or experimental cultures was released into the media under the conditions employed (5).

Short-term labeling of fibroblast cultures. In order to examine the effects of the IFNs on the kinetics of collagen synthesis and secretion, short-term labeling experiments were performed. For this purpose, confluent fibroblasts were incubated under control conditions or in the presence of 1,000 U/ml of aff-IFN-γ exactly as described above, and after 2 d they were labeled by the addition of 5 μCi/ml [^{14}C]proline to the culture media. Two plates from each set were harvested at 1, 3, and 6 h after labeling, and the media and cell layers were separated and processed as described above for the cultures labeled for 24 h.

Gel filtration. Chromatography of labeled proteins from the media and cell layers of control and IFN-treated cultures was performed on 1.5 × 85 cm columns of agarose A-5m, 200 to 400 mesh (Bio-Rad Laboratories, Richmond, CA) as previously described (12). Aliquots from each fraction were used to determine their [^{14}C]proline content. Peaks from the chromatograms were pooled and hydrolyzed for assay of their [^{14}C]hydroxyproline content.

Results

Effects of partially purified α-IFNs on protein and collagen synthesis by confluent human diploid fibroblasts. The effects of 100 and 1,000 U/ml of partially purified α-IFN on [^{14}C]proline incorporation and [^{14}C]hydroxyproline synthesis by confluent human fibroblast cultures are shown in Table I. The incorporation of [^{14}C]proline into media or cell layer proteins was not affected by α-IFN. In contrast, the total amount of [^{14}C]hydroxyproline synthesized by the cultures (media + cell layers) was 27.4 and 45.6% lower in cultures incubated with 100 U/ml and 1,000 U/ml, respectively, when compared with control cultures. These results also show that the decrease in media [^{14}C]hydroxyproline caused by IFNs was not due to impaired secretion and subsequent intracellular accumulation of the newly synthesized collagen.

Table I. Effect of Partially Purified α-IFN on [^{14}C]Proline Incorporation and [^{14}C]Hydroxyproline Synthesis by Confluent Cultures of Normal Human Diploid Fibroblasts

		IFN treated	
	Control	100 U/ml	1,000 U/ml
Media			
Total ^{14}C incorporation ($dpm \times 10^{-3}$)	36.4	33.8	34.6
[^{14}C]hydroxyproline synthesis ($dpm \times 10^{-3}$)	8.1	5.8	4.3
Degree of hydroxylation (%)*	22.2	17.1	12.4
Cell layers			
Total ^{14}C incorporation ($dpm \times 10^{-3}$)	32.8	31.4	33.2
[^{14}C]hydroxyproline synthesis ($dpm \times 10^{-3}$)	1.4	1.1	1.0
Degree of hydroxylation (%)*	4.3	3.5	3.1
Total (media + cell layers)			
Total ^{14}C incorporation ($dpm \times 10^{-3}$)	69.3	65.3	67.8
[^{14}C]hydroxyproline synthesis ($dpm \times 10^{-3}$)	9.5	6.9	5.3
Degree of hydroxylation (%)*	13.7	10.6	7.8

Cultures of confluent human diploid fibroblasts were incubated in control medium or in media containing 100 or 1,000 U/ml of partially purified α-IFN for 72 h and then labeled for 24 h with [^{14}C]proline as described in Methods. Labeled proteins in the media and cell layers were then analyzed for total ^{14}C incorporation and [^{14}C]hydroxyproline content. The values shown are averages of two cultures which differed from each other by <10%.

* Degree of hydroxylation represents [^{14}C]hydroxyproline/total [^{14}C] × 100.

Effects of various IFN preparations on protein and collagen biosynthesis by confluent human diploid fibroblasts. Various IFN preparations standardized to contain 1,000 U/ml of antiviral activity were tested (11). The results shown in Table II demonstrate that several of the IFNs studied caused significant inhibition of fibroblast collagen biosynthesis. The degree of inhibition, however, was not uniform with all the preparations. Confirming the results shown in Table I, the partially purified α-IFN caused a 56.5% decrease in the degree of hydroxylation of the media macromolecules compared with control cultures. Most of the preparations of HPLC-purified α-IFN studied were not inhibitory except for IL-null, which caused a 30.3% decrease. The preparation of IFN-A studied showed moderate inhibition (38.8%). In contrast, affinity purified γ-IFN showed a potent inhibitory effect since it caused a 71% decrease in the degree of hydroxylation of the media proteins.

Table II also shows that total ^{14}C incorporation was not significantly affected by the IFN preparations, indicating that the inhibition of collagen synthesis was not due to a general toxic effect or to nonspecific inhibition of protein synthesis. Furthermore, cell viability was >90% in all the cultures, irrespective of whether they contained IFNs. The active IFN preparations did not affect fibroblast proliferation as shown by cell counts (212×10^{-3} cells/plate in control cultures vs. 216×10^{-3} in cultures incubated with 1,000 U/ml of partially purified α-IFN and 262×10^{-3} in cultures incubated with 1,000 U/ml of affinity purified γ-IFN).

Table II. Effects of Various IFN Preparations on [^{14}C]Proline Incorporation and [^{14}C]Hydroxyproline Synthesis by Confluent Cultures of Normal Human Dermal Fibroblasts

Sample added to cultures	^{14}C-proline	[^{14}C]hydroxyproline	Degree of hydroxylation*
	dpm $\times 10^{-2}$	*dpm* $\times 10^{-2}$	%
None (control)	127.6	30.2	23.7
Partially purified α-IFN	111.7	11.5	10.3
HPLC-purified α-IFN			
IL-b1	133.6	31.8	23.8
IL-b2	117.8	28.8	24.5
IL-b3	145.3	33.0	22.7
IL-c1	116.7	25.1	21.5
IL-c2	130.5	31.2	23.9
IL-c3	101.4	21.5	21.2
IL-d1	152.4	44.2	29.0
IL-d2	128.2	31.8	24.8
IL-null	92.7	15.3	16.5
IFN-A	123.9	17.9	14.5
Aff-IFN-γ	137.4	9.44	6.9

Cultures of confluent human diploid fibroblasts were incubated in control medium or in medium containing 1,000 U/ml of the various IFN preparations and processed as described in Table I. Only media samples were analyzed. The values shown are averages of two cultures which differed from each other by <10%.
* Degree of hydroxylation represents [^{14}C]hydroxyproline/total [^{14}C] $\times$ 100.

Dose-response studies. To determine if the effects of IFN were dose dependent, various concentrations ranging from 0 to 1,000 U/ml of partially purified α-IFN, IFN-A, and aff-IFN-γ were tested. The results shown in Fig. 1 demonstrate that increasing concentrations of the three IFN preparations caused a progressive inhibition of fibroblast collagen production. The aff-IFN-γ was the most potent since it caused a 50% inhibition of collagen biosynthesis at a concentration of only 100 U/ml, and maximum inhibition (72%) was observed at a concentration of 500 U/ml. The partially purified α-IFN was less potent, since a concentration of 500 U/ml was required to produce a 50% decrease in [^{14}C]hydroxyproline synthesis, and the maximal inhibition observed was only 55%. The preparation of IFN-A showed less inhibitory effect since a significant inhibition (22%) was demonstrated only at the highest concentration tested (1,000 U/ml).

Chromatography of labeled proteins. SDS-agarose chromatography of the labeled proteins from the media of control cultures and from cultures labeled in the presence of 1,000 U/ml of partially purified α-IFN is shown in Fig. 2. In the chromatogram from control medium, a predominant peak containing >50% of the total radioactivity was found in the position of elution of standard dermal fibroblast procollagen (peak *B*). The remaining radioactivity was found in three other peaks of varying molecular weight (peaks *A*, *C*, and *D*). In contrast, in the sample from the IFN-treated culture, only 17% of the radioactivity in the chromatogram was in the procollagen peak. To identify the collagenous proteins in the chromatograms and to determine their degree of hydroxylation, four regions of each chromatogram were pooled as indicated in Fig. 2 and

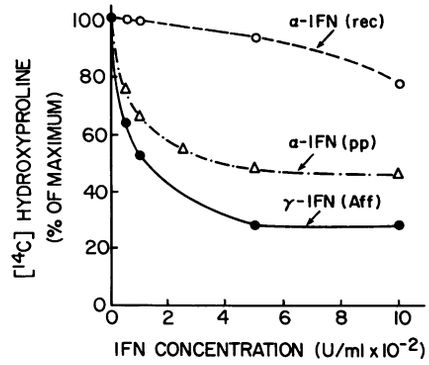

Figure 1. Dose response of inhibition of fibroblast collagen synthesis by IFNs. Confluent cultures of normal human dermal fibroblasts were incubated with increasing concentrations of partially purified (pp) α-IFN (Δ), IFN-A (rec) (O), or aff-IFN-γ (AFF) ($\bullet$), and the amount of collagen synthesized in each culture was determined as described in Methods. Each value represents the average of two cultures.

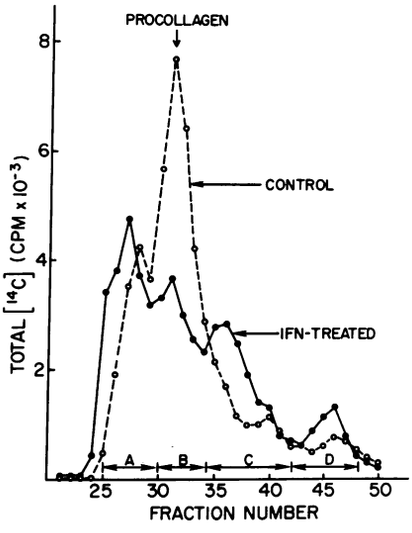

Figure 2. Gel filtration chromatography of [^{14}C]proline-labeled media proteins from control and IFN-treated fibroblast cultures. Confluent cultures of normal human dermal fibroblasts were incubated under control conditions (○) or with 1,000 U/ml partially purified α-IFN (●) and labeled with [^{14}C]proline as described in Methods. Samples of media from each culture were chromatographed on SDS-agarose columns as described previously (12). Aliquots were used to determine total [^{14}C]proline content. Fractions were pooled as shown, and their [^{14}C]hydroxyproline content was assayed.

analyzed for their [^{14}C]hydroxyproline content. In the control sample, the [^{14}C]hydroxyproline content of the procollagen peak was 34.3%, whereas in the IFN sample it was 41%. These results indicate that IFNs did not cause synthesis of underhydroxylated collagen molecules. The other prominent peaks (*A* and *C*) from both chromatograms contained a little [^{14}C]hydroxyproline, probably from some procollagen included in these pools. The small molecular weight peak (peak *D*), representing collagen degradation products, contained approximately equal amounts of [^{14}C]hydroxyproline in both cultures (367 dpm in control vs. 323 dpm in IFN-treated samples). These results indicate that the decrease in collagen production caused by IFN was not due to underhydroxylation or increased extracellular degradation of the newly synthesized collagen.

Effects of IFN on the short-term kinetics of collagen synthesis and secretion. To document further that the observed IFN effects were due to inhibition of collagen production rather than to extracellular degradation of the newly synthesized collagen, control or IFN-treated cultures were labeled for 1, 3, and 6 h, and the amounts of [^{14}C]hydroxyproline present in the cell layers and culture media were determined at each time interval after labeling. The results shown in Fig. 3 demonstrate that there was a linear increase with time in total [^{14}C]hydroxyproline production in control and IFN-treated cultures. However, the rate of total [^{14}C]hydroxyproline production was markedly decreased in the IFN-treated cultures compared with control cultures (<25% of control). The amount of [^{14}C]hydroxyproline present in the cell layers and culture

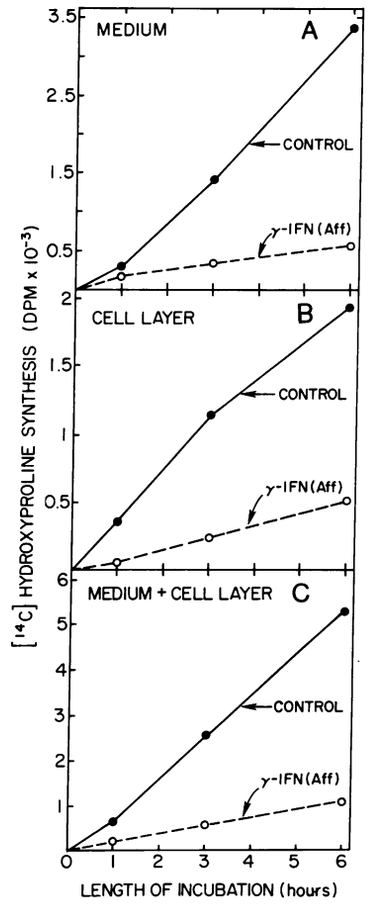

Figure 3. Short-term kinetics of collagen synthesis and secretion in control and IFN-treated fibroblast cultures. Confluent cultures of normal human dermal fibroblasts were incubated under control conditions (●) or with 1,000 U/ml aff-IFN-γ (Aff) (○) and labeled with [^{14}C]proline as described in Methods. Two plates from each set were harvested at 1, 3, and 6 h after labeling and the amount of [^{14}C]hydroxyproline present in the media and cell layers was determined. (*A*) Media; (*B*) cell layers; (*C*) total (cell layers + media).

media also displayed a linear increase with time under both experimental conditions, but it was substantially lower in the IFN-treated cultures at all time intervals studied. These results further demonstrate that the effect of IFN was due to an inhibition of net fibroblast collagen production and that the impairment of collagen secretion or increased extracellular degradation of the newly synthesized molecules did not significantly contribute to the overall IFN effects.

Discussion

The proximity of inflammatory cells with fibroblasts in areas of repair and early fibrosis has been noticed for some time, and the possibility that inflammatory cells can regulate fibroblast function has been given recent experimental attention (3–8). Of the various factors produced by inflammatory cells, IFNs have been some of the most extensively studied and characterized. In addition to their well known antiviral activity, IFNs can exert potent antiproliferative effects on a variety of cells, including fibroblasts (for review see reference 9). The results described here show that IFNs can inhibit collagen production by normal human diploid fibroblasts independently of any influence on cell replication. From the several IFN preparations

studied, the most potent inhibitory effects (up to 72% inhibition) were observed with aff-IFN-γ. The partially purified α-IFN was slightly less effective, although at higher concentrations it caused almost 60% inhibition of [^{14}C]hydroxyproline synthesis. In contrast, the preparations of HPLC-purified α-IFN were ineffective or caused only minor inhibition (<31% with 1,000 U/ml). Although the IFN-A showed substantially less collagen synthesis inhibitory activity than did the partially purified preparation, the observation that recombinant IFN molecules caused inhibition of fibroblast collagen synthesis indicates that this is a constitutive property of IFNs.

The marked decrease in collagen production by fibroblasts caused by IFNs did not appear to be due to underhydroxylation, impairment of secretion, or increased extracellular degradation of this macromolecule. From the present studies, however, it is impossible to determine if these effects reflect transcriptional or translational control of collagen synthesis or increased intracellular collagen degradation. Regardless of the mechanisms responsible for the IFN-induced decrease in fibroblast collagen production, our findings suggest that IFNs may play an important role in the regulation of collagen production.

Our results may also explain, at least in part, the observation that inflammatory reactions secondary to viral infections are not usually accompanied by significant tissue fibrosis. It can be hypothesized that in these circumstances, the high levels of IFNs produced may result in an inhibition of fibroblast proliferation as well as of collagen synthesis by these cells. On the other hand, it is tempting to speculate that alterations in IFN regulation or decreased sensitivity of fibroblasts to the modulatory effects of IFNs may play a role in the excessive and frequently progressive fibrosis observed in certain diseases associated with tissue infiltration by immunocompetent cells, such as scleroderma or idiopathic pulmonary fibrosis. In this regard it is interesting that γ-IFN, which may be produced during an immune response, was the most potent inhibitor of collagen synthesis. Finally, the marked inhibition of collagen production observed in fibroblast cultures incubated with relatively small concentrations of IFNs suggests the possibility that IFNs can be used as therapeutic agents to prevent the excessive fibrotic response frequently associated with these diseases.

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