Vol. 123, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1984 Pages 365-372

TRANSCRIPTIONAL CONTROL OF HUMAN DIPLOID FIBROBLAST COLLAGEN SYNTHESIS BY γ-INTERFERON

Joel Rosenbloom, George Feldman, Bruce Freundlich and Sergio A. Jimenez*

Rheumatology Section, Department of Medicine, School of Medicine, and
The Center for Oral Health Research, School of Dental Medicine,
University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received July 18, 1984

SUMMARY: Recombinant γ-interferon (rec γ-IFN) caused potent inhibition of collagen synthesis by cultured confluent human diploid fibroblasts in a dose-dependent manner. Gel electrophoresis of the newly synthesized proteins from the culture media of rec γ-IFN-treated fibroblasts demonstrated a selective depression of procollagen without a significant change in non-collagenous proteins. Dot blot hybridization to a Type I procollagen cDNA probe showed that the inhibition of collagen production was accompanied by a decrease in the levels of collagen mRNA. These results indicate that rec γ-IFN is capable of exerting transcriptional modulation of collagen biosynthesis and suggest that it may play an important role in regulation of normal and pathologic fibrogenesis.

Normal fibroblasts possess delicate regulatory mechanisms that permit the production of sufficient collagen to meet the demands of the extracellular matrix during dynamic processes such as development, differentiation and repair (1). Abnormalities in collagen regulation may be responsible for the excessive and frequently progressive fibrosis characteristic of certain diseases such as scleroderma (2) and pulmonary fibrosis. Collagen production can be controlled by three general mechanisms: a) modulation of the steady state level of mRNA; b) control of mRNA translation; and c) variation in the fraction of collagen which is degraded intracellularly. In addition, extra-fibroblastic factors may influence the rates of collagen synthesized by these cells. For example, the role of inflammatory cells on regulation of fibroblast proliferation and collagen synthesis has recently been emphasized (3-8). Because interferons (IFN) are important soluble products of cells

*To whom correspondence should be addressed at 570 Maloney Bldg., 3600 Spruce St., Phila., PA 19104.

0006-291X/84 $1.50

involved in inflammatory reactions and are capable of influencing the proliferation of various fibroblastic cell lines (9), we have examined the possibility that IFN may also modulate fibroblast collagen synthesis independently of their effects on cell proliferation. For this purpose, we have studied the effects of recombinant γ-interferon (rec γ-IFN) on collagen synthesis employing confluent dermal fibroblasts to dissociate the anti-proliferative effects of IFN from those related to regulation of collagen synthesis in stationary-phase cells. We found that rec γ-IFN caused a concentration-dependent inhibition of fibroblast collagen synthesis. Employing dot hybridization to a human procollagen cDNA probe, we found that this effect was accompanied by greater than 50% decrease in the levels of Type I collagen mRNA indicating that γ-IFN was capable of exerting transcriptional modulation of collagen synthesis in these cells. These results suggest that IFN may play an important role in regulation of collagen production particularly under pathologic conditions characterized by accumulation of inflammatory cells in the affected tissues.

MATERIALS AND METHODS

A preparation of recombinant γ-IFN was kindly supplied to us by Dr. Sidney Petska (Hoffman-LaRoche, Nutley, NJ) and was standardized to contain 1000 U/ml of anti-viral activity based on the cytophatic inhibition assay described by Rubenstein et. al. (10).

Fibroblast Cultures and Labeling Conditions. Fibroblasts were obtained from skin biopsies from normal individuals as described previously (11), and were cultured in Eagle's MEM supplemented with 10% fetal calf serum in an atmosphere of 5% CO_2 for 5 days to allow the cultures to reach confluency. Previous experiments demonstrated that after confluency, 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 rec γ-IFN, 5% fetal calf serum and 50 μg/ml ascorbate in Eagle's MEM were added. After 48 h the media was replaced with fresh media containing in addition from 1 to 4 μCi/ml [^{14}C]-proline and 100 μg/ml β-aminoproprionitrile, and the incubation was continued for 24 h.

Analyses of Labeled Proteins. After the 24 h of labeling, 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 amount of radioactive hydroxyproline synthesized by the cultured fibroblasts was measured by a specific chemical assay (12). In these experiments only the culture media were studied since as shown previously, greater than 80% of total collagen synthesized by the control or experimental cultures was released into the media under the conditions employed (5).

Gel Electrophoresis. Electrophoresis of labeled proteins from the media of control and IFN-treated cultures was performed on 5% polyacrylamide slab gels in

SDS. After electrophoresis the gels were processed for fluorography and the relative proportions of radioactivity in each band were calculated from densitometric scanning of the fluorographs.

Preparation of RNA and Dot-Blot Hybridization. Fibroblast mRNA was prepared as described previously (13). Briefly, confluent fibroblasts in T75 culture flasks were suspended and washed twice with phosphate buffered saline. Ten million cells in each flask were then lysed with 2 ml of a solution containing 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% SDS and 100 μg/ml proteinase K. The mixture was homogenized in a Dounce homogenizer 10 times and incubated at 40^{o} for 1 hr. After centrifugation of the mixture at 15,000 g for 20 min, the RNA in the supernatant was precipitated by the addition of two volumes of ethanol, and then dissolved in 0.5 ml of 0.1 mM EDTA. 0.5 to 1.0 μg/ml RNA were applied to nitrocellulose (NTC) which had been previously equilibrated with 10 x SSC using a BRL Hybridot apparatus as described by Thomas (21). Each well was washed with 150 μl of 10 x SSC, the NTC filter was baked at 80^{o}C for 2 hr, and then hybridized as described (14) to the Hf667 human cDNA clone, which corresponds to the carboxy-terminal half of the pro-α_1(I) mRNA (15). The cDNA clone was nick-translated to a specific activity of 1-3 x 10^{8} cpm/μg DNA using a kit supplied by BRL Laboratories. This clone has been shown to hybridize specifically to α_1(I) mRNA by Northern hybridization (22).

RESULTS

Effects of Recombinant γ-IFN on Protein and Collagen Synthesis by Confluent Human Diploid Fibroblasts. The effects of rec γ-IFN on [^{14}C]-proline incorporation and [^{14}C]-hydroxyproline synthesis by confluent human fibroblast cultures are shown in Table I. It was found that the amount of [^{14}C]-hydroxyproline synthesized in

TABLE I

EFFECT OF rec γ-IFN ON [^{14}C]-PROLINE INCORPORATION AND [^{14}C]-HYDROXYPROLINE SYNTHESIS BY CONFLUENT CULTURES OF NORMAL HUMAN DIPLOID FIBROBLASTS

	CONTROL	rec γ-IFN-TREATED	
		100 U/ml	1000 U/ml
Total [^{14}C]- incorporation	23.1	18.8 (81.3)	16.5 (71.4)
(dpm x 10^{-3})	22.5	18.5 (82.2)	15.2 (67.6)
[^{14}C]-hydroxyproline synthesis	4.36	2.04 (46.8)	1.16 (26.6)
(dpm x 10^{-3})	4.30	2.36 (54.9)	1.32 (30.7)

Confluent human diploid fibroblast cultures were incubated in control medium or in media containing 100 or 1000 U/ml of rec γ-IFN for 72 h and then labeled for 24 h with [^{14}C]-proline. Labeled proteins in the media were then analyzed for [^{14}C]--incorporation and [^{14}C]-hydroxyproline content. The values shown in parenthesis represent the percent difference between control and experimental cultures.

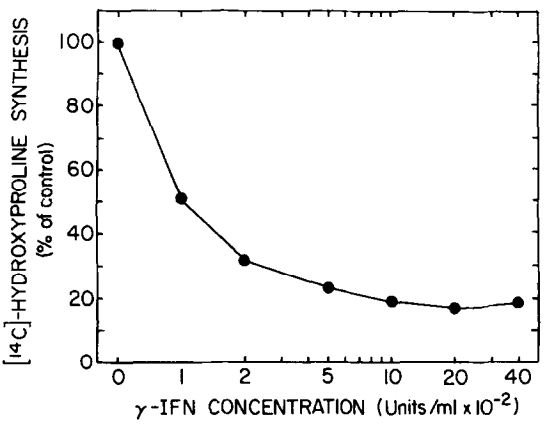

Fig. 1. Dose-response of inhibition of fibroblast collagen synthesis by rec γ-IFN. Confluent cultures of normal human dermal fibroblasts were incubated with increasing concentrations of γ-IFN (●---●) and the amount of collagen synthesized in each culture was determined as described in METHODS. Each value represents the average of two cultures.

cultures incubated with 100 U/ml and 1000 U/ml rec γ-IFN was strikingly lower than in control cultures (50% and 70% inhibition respectively). The marked decrease in [^{14}C]-hydroxyproline synthesis caused by rec γ-IFN was accompanied by only a modest reduction of [^{14}C]-proline incorporation (20% and 33% respectively) which can be accounted for by the inhibition of collagen synthesis.

Dose-response Studies. To examine if the effects of rec γ-IFN on [^{14}C]-hydroxyproline synthesis were dose-dependent, various concentrations ranging from 0 to 4000 U/ml were tested. The results shown in Fig. 1 demonstrate that increasing concentrations of rec γ-IFN caused a progressive inhibition of fibroblast collagen production. In these experiments, a concentration of 100 U/ml caused approximately 50% inhibition of [^{14}C]-hydroxyproline synthesis and maximum inhibition ($\simeq$ 80%) occured at a concentration of 500 U/ml.

Gel Electrophoresis and Autoradiography of Labeled Proteins From Fibroblast Cultures. To further characterize the effects of rec γ-IFN on fibroblast biosynthesis, the newly synthesized proteins from the culture media of fibroblasts incubated under control conditions or with 1000 U/ml rec γ-IFN were examined by electrophoresis on SDS-polyacrylamide slab gels and subsequent fluorography. As shown in Fig. 2(A), rec γ-IFN caused a selective decrease in the proportion of radioactivity migrating in the procollagen region without a discernible reduction in radioactivity

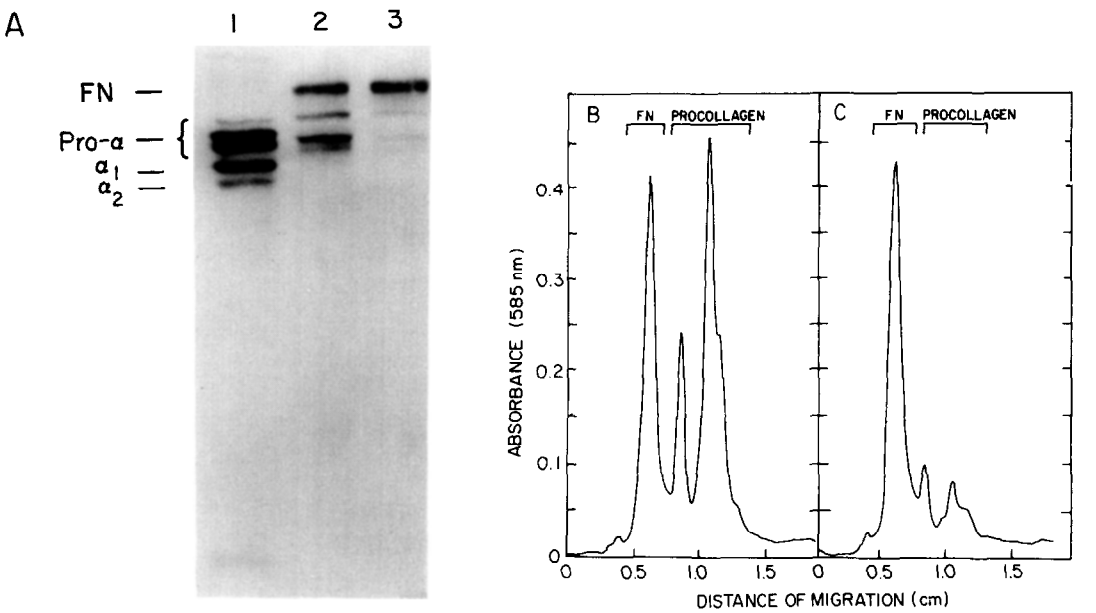

Fig. 2. Fluorograph of SDS-polyacrylamide slab gel electrophoresis of media proteins from control and rec γ-IFN-treated fibroblast cultures. Confluent cultures of normal human dermal fibroblasts incubated under control conditions or with 1000 U/ml rec γ-IFN were labeled with 4 μCi/ml [^{14}C]-proline as described in Methods. After 18 h labeling the media were removed and equal aliquots containing approximately 10,000 dpm were denatured in SDS-mercaptoethanol and electrophoresed in slab gels. Fluorographs of the gels were scanned at 585 nm in a densitometer and the areas under each peak calculated with a planimeter.

(A) Fluorographs of labeled media proteins. Lane 1: Standard chick tendon Type (I) procollagen and collagen. Lane 2: Medium from control cultures. Lane 3: Medium from rec γ-IFN-treated cultures.
(B) Densitometric scanning of fluorograph from control culture medium.
(C) Densitometric scanning of fluorograph from rec γ-IFN-treated culture medium.

migrating in the region of fibronectin. In addition, no detectable increase in radioactivity corresponding to collagen cleaved products was observed. When the relative proportions of fibronectin and procollagen were calculated from densitometric scanning of the fluorographs, it was found that in control cultures the ratio of fibronectin to procollagen was 0.61 (Fig. 2B). In contrast, in the rec γ-IFN-treated cultures this ratio increased 5.5-fold to 3.32 (Fig. 2C). These results indicate that rec γ-IFN caused selective inhibition of fibroblast collagen synthesis relative to non-collagenous proteins. The results also showed that the observed decrease in collagen production was not due to increased extracellular degradation of the newly synthesized molecules.

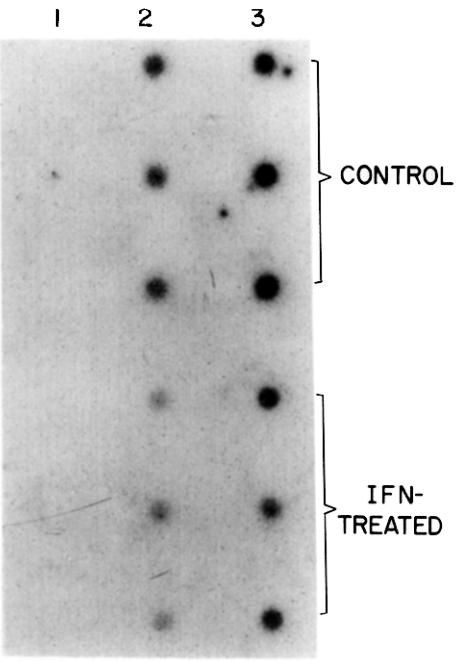

Fig. 3. Dot-blot hybridization of mRNA from control and rec γ-IFN-treated fibroblast cultures. mRNA was isolated from 10 x 10^6 control and rec γ-IFN-treated fibroblasts as described in Methods. Aliquots containing 50 μg and 100 μg mRNA were bound to NTC and dot-hybridized to the human cDNA clone Hf667 according to the method of Thomas (14). Lane 1: No mRNA. Lane 2: 50 μg mRNA. Lane 3: 100 μg mRNA.

Dot-Blot Hybridization. To further document that the effect of rec γ-IFN was due to inhibition of fibroblast collagen synthesis and to investigate the responsible mechanisms, the levels of specific Type I collagen mRNA were measured employing a human cDNA clone specific for pro-α_1(I) collagen. For this purpose, mRNA isolated from an equal number of control and γ-IFN treated fibroblast was dot-hybridized to the human cDNA clone Hf667 which corresponds to the carboxy-terminal half of pro-α_1(I) mRNA. The results shown in Fig. 3 and Table II demonstrate that there was greater than 50% reduction in specific Type I collagen mRNA in the γ-IFN treated cells.

DISCUSSION

The close proximity of inflammatory cells with fibroblasts in areas of repair and early fibrosis suggested the possibility that inflammatory cells can regulate fibroblast function (3-8). From the variety of factors produced by inflammatory

TABLE II

DENSITOMETRIC QUANTITATION OF SCANS FROM FLUOROGRAPHS OF COLLAGEN mRNA HYBRIDIZED TO Hf667 cDNA (TYPE I PROCOLLAGEN) PROBE

Area under each peak (mm^2)			
50 µg		100 µg	
Control	rec γ-IFN	Control	rec γ-IFN
99.3	44.5	209.0	111.6
89.0	30.3	194.8	109.0
79.3	29.6	167.7	80.6
89.2 ± 10.0	34.8 ± 8.4	190.5 ± 20.9	100.4 ± 17.2
$p < 0.005$		$p < 0.005$	

mRNA was isolated from 10 x 10^6 control and rec γ-IFN-treated fibroblasts and aliquots containing 50 µg and 100 µg RNA were dot-hybridized according to the method of Thomas (14). The fluorographs shown in Fig. 3 were scanned at 585 nm and the areas under each peak quantitated with a planimeter.

cells, IFN have been some of the most extensively studied and characterized. In addition to their well known anti-viral activity, IFN can exert potent anti-proliferative effects on a variety of cells including fibroblasts (9). The results described here show that IFN can inhibit collagen production by normal human diploid fibroblasts independently of any influence on cell replication. The observation that [^{14}C]-proline incorporation was only moderately decreased by rec α-IFN coupled with the demonstration that the ratio of fibronectin to procollagen markedly increased in the IFN-treated cultures indicate that IFN selectively inhibited collagen synthesis. The results of the dot-blot hybridization and subsequent densitometric analysis clearly demonstrated that the levels of pro-α_1(I) collagen mRNA were decreased in the γ-IFN treated cells. This finding suggests that the observed decrease in collagen production caused by γ-IFN is due to a corresponding decrease in collagen mRNA. These results also suggest that γ-IFN decreases the rate at which the procollagen α_1(I) gene is transcribed since in most systems transcription has been shown to be the major level of control. Further experiments are necessary to

determine whether such decrease in mRNA is responsible for the entire decrease in collagen synthesis and whether there is coordinate decrease in other mRNA species.

ACKNOWLEDGEMENTS We thank Dr. Jeanne Myers for providing the human cDNA clone, Hf667. We gratefully acknowledge the technical assistance of Mary Dixon and the assistance of Esther Lobb in preparation of this manuscript. Supported by NIH grant AM 19106. Dr. Bruce Freundlich is recipient of NIH Clinical Investigator Award AM-01174.

REFERENCES

1. Pinnell, S.R. J. Invest. Dermatol. 79(suppl. 1):73, 1982.
2. LeRoy, E.C. J. Clin. Invest. 54:880, 1974.
3. Johnson R.L., and M. Ziff. J. Clin. Invest. 48:240, 1976.
4. Wahl, S.M., L. M. Wahl, and J.B., McCarthy. J. Immunol. 121:942, 1978.
5. Jimenez, S.A., W. McArthur, and J. Rosenbloom. J. Exp. Med. 150:1421, 1979.
6. Hibbs, M.S., A.E. Postlethwaite, C.L. Mainardi, J.M. Seyer, and A.H. Kang. J. Exp. Med. 157:47, 1983.
7. Hart, P.H., L.W. Powell, W.G.E. Cooksley, and J.W. Halliday. Scand. J. Immunol. 18:41, 1983.
8. Nielson, E.G., S.M. Phillips, and S.A. Jimenez. J. Immunol. 128:1484, 1982.
9. Brouty-Boye, D. Lymphokine Reports 1:99, 1980.
10. Rubinstein, S., P.C. Familletti, and S. Pestka. J. Virol. 37:755, 1981.
11. Bashey, R.I., S.A. Jimenez, and J.S. Perlish. J. Molec. Med. 2:153, 1977.
12. Juva, K., and D.J. Prockop. Anal. Biochem. 15:77, 1966.
13. Burnett, W. and Rosenbloom, J.: Biochem. Biophys. Res. Commun. 86:478, 1979.
14. Thomas, P.S.: Proc. Nat. Acad. Sci. USA 77:5201, 1980.
15. Myers, J.C., L.A. Dickson, W.J. deWet, M.P. Bernard, M.-L. Chu, M. DiLiberto, G. Pepe, F.O. Sangiorgi, F. Ramirez. J. Biol. Chem. 257:10128, 1983.