

**Tumor necrosis factor-alpha and interferon-gamma suppress the activation of human type I collagen gene expression by transforming growth factor-beta 1. Evidence for two distinct mechanisms of inhibition at the transcriptional and posttranscriptional levels.**

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*J Clin Invest.* 1990;**86**(5):1489-1495. <https://doi.org/10.1172/JCI114866>.

**Research Article**

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# Tumor Necrosis Factor- $\alpha$ and Interferon- $\gamma$ Suppress the Activation of Human Type I Collagen Gene Expression by Transforming Growth Factor- $\beta$ 1

## Evidence for Two Distinct Mechanisms of Inhibition at the Transcriptional and Posttranscriptional Levels

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### Abstract

Regulation of human type I procollagen gene expression was studied in cultured fibroblasts both at the transcriptional and posttranscriptional level. Transcriptional regulation was examined in cultures transfected with a human pro $\alpha$ 2(I) collagen promoter/reporter gene (chloramphenicol acetyltransferase) construct, while posttranscriptional regulation was assessed by parallel determinations of type I procollagen mRNA steady-state levels. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) elicited a marked,  $\sim$  5–23-fold, enhancement of pro $\alpha$ 2(I) collagen promoter activity, which was accompanied by an elevation of type I procollagen mRNA levels. This enhancement of gene expression was suppressed by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ), as determined at mRNA steady-state level, but two distinct mechanisms were involved. TNF- $\alpha$  suppressed the pro $\alpha$ 2(I) collagen promoter activity, whereas IFN- $\gamma$  had only a minimal effect at transcriptional level. The effects of TNF- $\alpha$  and IFN- $\gamma$  were synergistic, suggesting that combination of these two factors may potentially provide pharmacologic means to counteract tissue deposition of collagen in diseases involving TGF- $\beta$ . (*J. Clin. Invest.* 1990; 86:1489–1495.) Key words: collagen gene expression • growth factors • fibrotic diseases

### Introduction

The synthesis and accumulation of connective tissue components of the extracellular matrix play a crucial role in biological situations involving tissue development, homeostasis, and repair. An aberration of these processes may also lead to development of pathological conditions, as in case of fibrotic diseases (1, 2). Recently, a number of polypeptide growth factors have been shown to modulate the growth and migration of connective tissue derived cells, as well as the synthesis of extracellular matrix components, such as the collagens (3–5). For example, proliferation of fibroblasts, the mesenchymal cell responsible for extracellular matrix production in the skin and other connective tissues, has been shown to be markedly stimulated by several growth factors, including transforming

growth factor  $\beta$ 1 (TGF- $\beta$ 1)<sup>1</sup> (5, 6). The expansion of fibroblast populations in the tissues may then contribute to deposition of extracellular matrix components in the affected organs.

Certain growth factors have also been shown to enhance the expression of the extracellular matrix genes by individual connective tissue cells. In particular, TGF- $\beta$  has been shown to be a potent stimulator of collagen synthesis by fibroblasts in a variety of experimental conditions both in vivo and in vitro (5, 7). Specifically, TGF- $\beta$  enhances the synthesis of collagens type I and III, as well as of fibronectin, an abundant noncollagenous glycoprotein (7–10). Enhanced collagen and fibronectin gene expression by TGF- $\beta$  has been shown to be accompanied by similar increases in the corresponding mRNA steady-state levels, suggesting transcriptional activation of the corresponding genes (7–10). A previous study using a chimeric mouse pro $\alpha$ 2(I) collagen promoter/reporter gene construct has shown enhancement of transcriptional activity by TGF- $\beta$  in mouse NIH-3T3 cells (11). In addition to TGF- $\beta$ , other polypeptide growth factors are able to modulate collagen synthesis. For example, interleukin 1 has been shown to enhance collagen production by dermal fibroblasts at pretranslational level (12–14). In contrast, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) suppress collagen synthesis (15–18), although platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) have apparently little, if any, effect in vitro (7). Relatively little is known of the mechanisms by which these growth factors modulate the expression of collagen genes.

Several recent studies have provided evidence that activation of type I collagen gene expression by fibroblasts plays a crucial role in the development of dermal fibrosis in diseases, such as scleroderma, eosinophilic fasciitis, and keloids (19–22). The role of TGF- $\beta$  in the pathogenesis of the fibrotic conditions has been emphasized, but relatively little is known about interactions of TGF- $\beta$  and other growth factors with respect to collagen gene expression.

We have recently demonstrated that the 3.5-kb upstream sequence of the pro $\alpha$ 2(I) collagen gene, linked to the chloramphenicol acetyltransferase (CAT) reporter gene, contains all elements necessary for high, tissue-specific transcription (23). In contrast, transfection experiments with a human pro $\alpha$ 1(I) collagen promoter/CAT construct did not provide similar evidence of tissue specific expression (23). Thus, in this study we utilized the pro $\alpha$ 2(I) collagen promoter/CAT plasmid,

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Received for publication 13 April 1990 and in revised form 22 June 1990.

*J. Clin. Invest.*

© The American Society for Clinical Investigation, Inc.

0021-9738/90/11/1489/07 \$2.00

Volume 86, November 1990, 1489–1495

1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; CAT, chloramphenicol acetyltransferase; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF-I, insulin-like growth factor I; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor.

pMS-3.5/CAT, as a tool to study transcriptional regulation of type I collagen gene expression. Specifically, we have examined the potential interactions of TGF- $\beta$ 1 with TNF- $\alpha$  and IFN- $\gamma$  in modulating collagen gene expression. We have also examined four other polypeptide growth factors for their effects on human pro $\alpha$ 2(I) collagen promoter activity in cultured fibroblasts.

## Methods

**Transient transfections of cultured cells.** Human skin fibroblast cultures were established from tissue specimens obtained from cosmetic surgery procedures, and utilized in passages 3–8. The cell cultures were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 50  $\mu$ g/ml streptomycin. Mouse NIH-3T3 cells were purchased from American Type Culture Collection, Rockville, MD, and maintained in DME supplemented with 10% calf serum, 2 mM glutamine, and the antibiotics indicated above.

The cell cultures in late logarithmic growth phase were transfected with 10  $\mu$ g (NIH 3T3 cells) or 20  $\mu$ g (human skin fibroblasts) of plasmid DNA, pMS-3.5/CAT, which contains  $\sim$  3.5 kb of 5'-flanking DNA of human pro $\alpha$ 2(I) collagen gene linked to the CAT reporter gene (23). Transfections were performed with the calcium phosphate/DNA co-precipitation method, followed by 1 min (human skin fibroblasts) or 2 min (NIH-3T3 cells) of glycerol shock (15%) (24). After the glycerol shock, the cells were placed either in serum-free medium (NIH-3T3 cells) or in medium supplemented with 1% heat-inactivated fetal calf serum (human skin fibroblasts). 3 h later, the growth factors tested were added, and the incubations were continued for 40 h. After incubations with or without the growth factors, the cells were harvested and lysed by three cycles of freeze-thawing in 100  $\mu$ l of 0.25 M Tris-HCl, pH 7.8. The protein concentration of each extract was determined with a protein assay kit (BioRad Laboratories, Richmond, CA), and identical amounts (10–30  $\mu$ g) of protein from each cell extract in each experiment were used for parallel determinations of CAT activity using [ $^{14}$ C]chloramphenicol as substrate (25). The acetylated and non-acetylated forms of radioactive chloramphenicol were separated by thin-layer chromatography and visualized by autoradiography. The enzyme activity was quantitated by cutting out pieces of thin-layer chromatography plates containing different forms of [ $^{14}$ C]chloramphenicol, and the radioactivity was determined by liquid scintillation counting. The values were expressed as the percentage of [ $^{14}$ C]chloramphenicol converted to its acetylated forms per 10  $\mu$ g of cell extract protein. Parallel transfections were performed with pBSOCAT, a promoterless CAT construct (26), and with pSV2CAT, a construct containing SV40 early region promoter and SV40 enhancer linked to the CAT gene (25).

**Northern analyses.** Human skin fibroblast cultures incubated with or without varying growth factors in medium supplemented with 1% fetal calf serum for a period of 24 h were subjected to isolation of total cellular RNA, as described previously (27). RNA, 12  $\mu$ g per lane, was fractionated on 0.8% agarose gels, transferred to nylon filters (Zeta Probe, BioRad Laboratories), and immobilized by heating at 80°C for 30 min under vacuum. The filters were then prehybridized and hybridized with cDNA probes labeled radioactive by nick translation with [ $\alpha$ - $^{32}$ P]dCTP (28). After hybridizations at 42°C, the filters were washed in solutions with decreasing ionic strength and increasing temperature, and the final stringency of the washes was 0.1 $\times$  SSC at 65°C. The [ $^{32}$ P]cDNA-mRNA hybrids were visualized by autoradiography, and the corresponding steady-state levels of mRNAs were quantitated by scanning densitometry, using a HeNe laser scanner at 633 nm (LKB Produkter, Bromma, Sweden).

The following cDNAs were used for Northern hybridizations: For pro $\alpha$ 1(I) collagen mRNA, a 1.5-kb human cDNA (Hf677); for pro $\alpha$ 2(I) collagen mRNA, a 2.9-kb cDNA (Hf1132) (29, 30); for glyc-

eraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, a 1.3 kb rat cDNA (pRGAPDH13) (31).

**Growth factors and cytokines.** The following growth factors and cytokines were used in these studies. TGF- $\beta$ 1 was kindly provided by Dr. David R. Olsen, Collagen Corporation, Palo Alto, CA; human recombinant IFN- $\gamma$ , TNF- $\alpha$ , basic fibroblast growth factor (bFGF), mouse EGF, and porcine PDGF were purchased from Boehringer-Mannheim Biochemicals, Indianapolis, IN; human recombinant insulin-like growth factor I (IGF-I) was purchased from Collaborative Research, Bedford, MA.

In transient cell transfection experiments, the growth factors were added to the cell culture media 3 h after the glycerol shock, and the incubations were continued for an additional 40 h before assay of CAT activity. The transfection efficiencies in cultures incubated with the growth factors were checked by assay of relative plasmid copy numbers, as described previously (32). In cultures used for determination of mRNA steady-state levels by Northern analyses, the growth factors were added to the cultures at early confluency, and the incubations were continued for 24 h before isolation of total cellular RNA.

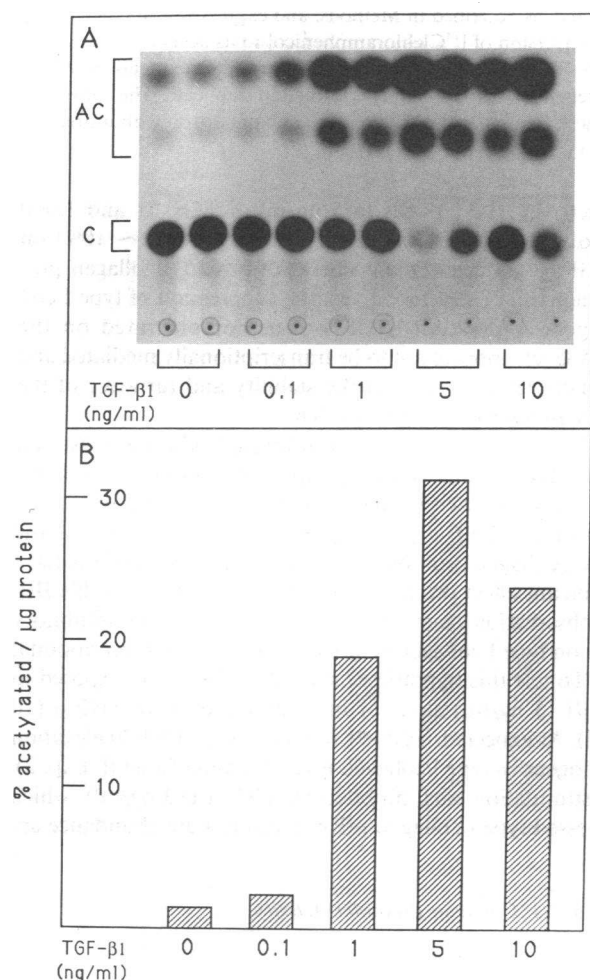
## Results

**Modulation of human pro $\alpha$ 2(I) collagen promoter activity by TGF- $\beta$ 1.** A human collagen promoter/reporter gene construct pMS-3.5/CAT, which contains 3.5 kb of 5'-flanking sequences of pro $\alpha$ 2(I) collagen (COLIA2) gene linked to the CAT gene, was previously shown to exhibit high, tissue-specific transcriptional activity (23). This chimeric construct was used in this study for transient cell transfection experiments to determine the effects of TGF- $\beta$ 1 on type I collagen promoter activity. Preliminary experiments showed that significant CAT activity could be detected in human skin fibroblasts and in mouse NIH-3T3 cells at 24–72 h of incubation after transfection (26). The effects of TGF- $\beta$ 1 were first tested by adding varying concentrations of this growth factor to the incubation medium of NIH-3T3 cells at 3 h after the glycerol shock. After an additional 40-h incubation, the cells were harvested and the CAT activity was measured as an index of the promoter activity. The results indicated that TGF- $\beta$ 1, in the concentration range of 0.1–10 ng/ml, significantly enhanced the activity of the pro $\alpha$ 2(I) collagen promoter in an apparently dose-dependent manner (Fig. 1 A). Quantitation of the data by liquid scintillation counting (see Methods) revealed that the highest promoter activity,  $\sim$  23-fold higher than found in the untreated control cells, was noted with 5 ng/ml of TGF- $\beta$ 1, while increasing the concentration to 10 ng/ml did not further enhance the activity (Fig. 1 B). Assay of plasmid copy number (32) in cultures incubated with and without TGF- $\beta$ 1 did not reveal any differences (data not shown). Thus, in subsequent experiments either 5 or 10 ng/ml of TGF- $\beta$ 1 was used.

Parallel transfections with the promoterless pBSOCAT construct (26) revealed minimal CAT activity in NIH-3T3 cells, and incubation of cells transfected with this construct with 10 ng/ml of TGF- $\beta$ 1 did not significantly increase the activity (Table I). Also, expression of pSV2CAT, a positive control plasmid containing SV40 early region promoter linked to the CAT gene (25), was unaffected by the addition of TGF- $\beta$ 1 (Table I). Thus, the enhancement of human pro $\alpha$ 2(I) collagen promoter activity appeared to be specific and to depend on the presence of TGF- $\beta$ 1 responsive elements within the 3.5 kb of sequence flanking the 5'-end of the pro $\alpha$ 2(I) collagen gene. In addition, these results suggested that TGF- $\beta$ 1 does not modulate the stability of CAT mRNA or protein, but that the in-

crease in CAT activity detected in transfected cells exposed to TGF- $\beta$ 1 reflects stimulation of the pro $\alpha$ 2(I) collagen promoter transcriptional activity.

To examine the concomitant effect of TGF- $\beta$ 1 on the endogenous expression of type I collagen genes, the pro $\alpha$ 1(I) and pro $\alpha$ 2(I) collagen mRNA abundance was determined. Incubation of cultured human skin fibroblasts with TGF- $\beta$ 1 (5 ng/ml) for a period of 24 h resulted in a marked increase in the steady-state levels of both these mRNAs (Fig. 2 and Table II). This increase was selective in that the steady-state levels of mRNA for GAPDH, a gene constitutively expressed in these cells (31), were not altered by TGF- $\beta$ 1 (Fig. 2). These results suggest that the expression of type I collagen genes is specifically upregulated by TGF- $\beta$ 1 at the transcriptional level.



**Figure 1.** Enhancement of type I procollagen promoter activity by TGF- $\beta$ 1 in transient cell transfections. Cultured NIH-3T3 cells were transfected with the human pro $\alpha$ 2(I) collagen promoter/CAT gene construct pMS-3.5/CAT, as indicated in Methods. 3 h after the glycerol shock, the cells were exposed to varying concentrations of TGF- $\beta$ 1 in serum-free medium for 40 h. The cells were then harvested, and CAT activity was determined as described in Methods. (A) Autoradiogram of the CAT assay depicting the separation of acetylated (AC) and unacetylated (C) forms of [ $^{14}$ C]chloramphenicol by a thin-layer chromatography. (B) Quantitation of CAT activity, expressed as the percentage of acetylated [ $^{14}$ C]chloramphenicol per 1.0  $\mu$ g of cell extract protein used for assay. The values are mean of duplicate cultures assayed as in A.

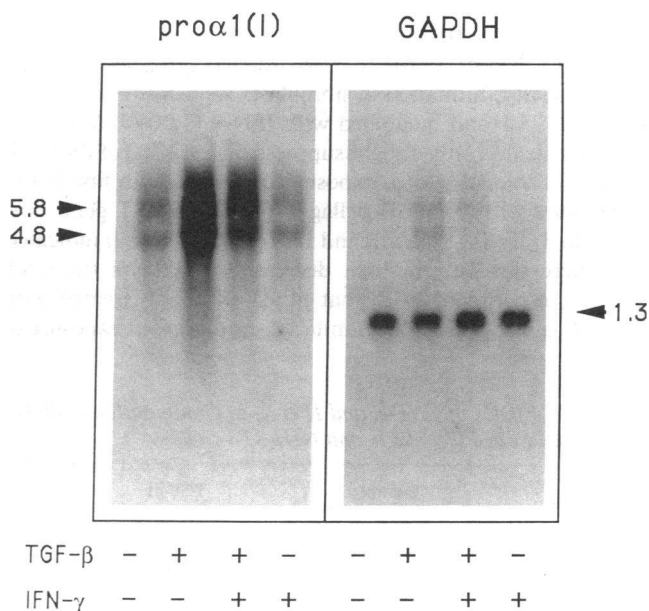
**Table I.** Selective Effect of TGF- $\beta$ 1 on Pro $\alpha$ 2(I) Collagen Promoter Activity in Transient Transfections of NIH-3T3 Cells

Plasmid	CAT activity	
	Control	TGF- $\beta$ 1 (10 ng/ml)
Pro $\alpha$ 2(I)/CAT	0.86 $\pm$ 0.26 (1.00)	4.09 $\pm$ 0.84 (4.76)
pSV2CAT	6.40 $\pm$ 1.68 (1.00)	6.30 $\pm$ 1.76 (0.98)
pBS0CAT	0.15 $\pm$ 0.04 (1.00)	0.19 $\pm$ 0.08 (1.27)

Mouse NIH-3T3 cell cultures were transfected with 10  $\mu$ g of the plasmid indicated using CaPO $_4$ /DNA co-precipitation method, followed by a 2-min glycerol shock. The cultures were placed in serum-free medium, and 3 h later, the cells were exposed to 10 ng/ml TGF- $\beta$ 1 for 40 hours. The cells were then harvested and CAT activity was determined, as described in Methods. The values represent the percentage of conversion of [ $^{14}$ C]chloramphenicol to its acetylated forms per 10  $\mu$ g of cell extract protein. The values are mean $\pm$ SEM of three independent experiments, each performed using duplicate cultures. The values in parentheses represent the change in CAT activity elicited by TGF- $\beta$ 1 in relation to the corresponding untreated controls with each plasmid.

**Effects of TNF- $\alpha$  and IFN- $\gamma$  on type I collagen gene expression.** To further elucidate the regulation of the transcriptional activity of type I collagen genes, we examined the effects of two well-known inhibitors of collagen production, viz. TNF- $\alpha$  and IFN- $\gamma$  (15–18).

Incubation of human skin fibroblasts with IFN- $\gamma$  (1,000 U/ml) together with TGF- $\beta$ 1 (5 ng/ml) clearly suppressed



**Figure 2.** Assay of pro $\alpha$ 1(I) collagen mRNA steady-state levels in human skin fibroblast cultures incubated for 24 h in medium supplemented with 1% fetal calf serum, TGF- $\beta$ 1 (5 ng/ml), and/or IFN- $\gamma$  (1,000 U/ml). Total RNA was isolated and analyzed (12  $\mu$ g per lane) by Northern hybridizations with a human pro $\alpha$ 1(I) collagen (left) or a GAPDH (right) cDNA. The sizes of the two polymorphic mRNA transcripts (5.8 and 4.8 kb) for human pro $\alpha$ 1(I) collagen and the size for GAPDH mRNA (1.3 kb) are indicated.

**Table II. Type I Collagen mRNA Abundance in Human Skin Fibroblasts Treated with TGF- $\beta$ 1 Alone or in Combination with IFN- $\gamma$  and/or TNF- $\alpha$**

Exp. 1*	Type I collagen mRNA levels			
	Control	TGF- $\beta$ 1	TGF- $\beta$ 1 + IFN- $\gamma$	IFN- $\gamma$
pro $\alpha$ 1(I)	1.80 (1.00)	10.50 (5.83)	4.22 (2.34)	1.34 (0.74)
pro $\alpha$ 2(I)	1.88 (1.00)	5.94 (3.16)	1.85 (0.98)	1.30 (0.69)

Exp. 2*	Type I collagen mRNA levels			
	Control	TGF- $\beta$ 1	TGF- $\beta$ 1 + TNF- $\alpha$	TGF- $\beta$ 1 + TNF- $\alpha$ + IFN- $\gamma$
pro $\alpha$ 1(I)	1.34 (1.00)	6.38 (4.76)	1.55 (1.16)	1.67 (1.24)
pro $\alpha$ 2(I)	0.32 (1.00)	0.71 (2.22)	0.34 (1.06)	0.45 (1.41)

Human skin fibroblasts were exposed to TGF- $\beta$ 1 (5 ng/ml) alone or in combination with IFN- $\gamma$  (1,000 U/ml) and/or TNF- $\alpha$  (10 ng/ml) for 24 h in DME supplemented with 1% FCS. The values represent absorbance values scanned from X-ray films of Northern blots (see Fig. 2), corrected for GAPDH mRNA in the same RNA preparations. The values in parentheses represent -fold activation of gene expression in treated cultures in comparison to the untreated controls. \* The same filters were successively hybridized with pro $\alpha$ 1(I) and pro $\alpha$ 2(I) collagen cDNAs. Thus, the values for the corresponding mRNAs are not directly comparable.

pro $\alpha$ 1(I) and pro $\alpha$ 2(I) collagen mRNA levels approximately by 60–70% as compared to cells incubated with TGF- $\beta$ 1 alone (Fig. 2 and Table II).

To test the effect of IFN- $\gamma$  on pro $\alpha$ 2(I) collagen transcriptional activity, human skin fibroblasts were transfected with pMS-3.5 CAT and incubated with IFN- $\gamma$  (1,000 U/ml). The results indicated only a slight suppression ( $\sim$  17%) of the CAT activity (Table III). Also, exposure of human skin fibroblasts transfected with pro $\alpha$ 2(I) collagen promoter/CAT gene construct to TGF- $\beta$ 1 (5 ng/ml) and IFN- $\gamma$  (1,000 U/ml) indicated that there was only a slight decrease ( $\sim$  30%) in the CAT activity in comparison to that noted with cells treated with TGF- $\beta$ 1 alone (Table IV). Similarly, simultaneous exposure of

**Table III. Effects of TNF- $\alpha$  and IFN- $\gamma$  on the Expression of pro $\alpha$ 2(I)/CAT and pSV2CAT Plasmids in Human Skin Fibroblasts as Determined by CAT Activity**

Plasmid	CAT activity			
	Control	TNF- $\alpha$	IFN- $\gamma$	TNF- $\alpha$ + IFN- $\gamma$
pro $\alpha$ 2(I)CAT	3.70 $\pm$ 0.40 (1.00)	0.62 $\pm$ 0.12 (0.17)	3.07 $\pm$ 0.27 (0.83)	0.74 $\pm$ 0.21 (0.20)
pSV2CAT	42.22 $\pm$ 2.60 (1.00)	41.44 $\pm$ 8.56 (0.98)	21.15 $\pm$ 6.80 (0.50)	24.62 $\pm$ 5.20 (0.58)

Human skin fibroblasts were transfected with 20  $\mu$ g of pro $\alpha$ 2(I)CAT or pSV2CAT, and the cultures were incubated with TNF- $\alpha$  (10 ng/ml) or IFN- $\gamma$  (1,000 U/ml) alone or in combination, as indicated, in DME supplemented with 1% fetal calf serum. CAT activity was determined, as described in Methods, and expressed as the percentage of conversion of [ $^{14}$ C]chloramphenicol to its acetylated derivatives per 10  $\mu$ g of cell extract protein. The results are mean $\pm$ SEM of two independent experiments performed in duplicate. The values in parentheses represent the -fold change in comparison with untreated control cultures.

transfected NIH-3T3 cells to 5 ng/ml of TGF- $\beta$ 1 and 1,000 U/ml of IFN- $\gamma$  had a slightly suppressing effect ( $\sim$  10%) on the TGF- $\beta$ 1 induced enhancement of pro $\alpha$ 2(I) collagen promoter activity (Table IV). Thus, the suppression of type I collagen gene expression by IFN- $\gamma$ , as demonstrated on the mRNA level, appears not to be transcriptionally mediated and potentially revolves around the stability and turnover of the newly synthesized collagen mRNA.

TNF- $\alpha$  was similarly tested in human skin fibroblast and mouse NIH-3T3 cell cultures. Exposure of human skin fibroblasts transfected with pMS-3.5/CAT to 10 ng/ml of human recombinant TNF- $\alpha$  resulted in  $\sim$  83% inhibition of the pro $\alpha$ 2(I) collagen promoter activity, while TNF- $\alpha$  did not have a significant effect on the expression of pSV2CAT (Table III). This observation suggested that TNF- $\alpha$  exerts its inhibitory effects on type I collagen gene expression at the transcriptional level. To test this hypothesis, skin fibroblasts were exposed to TGF- $\beta$ 1 (5 ng/ml) alone or in combination with TNF- $\alpha$  (10 ng/ml). As expected, TGF- $\beta$ 1 induced a 2.2–4.8-fold elevation in endogenous type I collagen mRNA levels (Table II, Exp. 2). This stimulation was inhibited by TNF- $\alpha$  (10 ng/ml), which suppressed type I collagen mRNA steady-state abundance ap-

**Table IV. Effects of TNF- $\alpha$  and IFN- $\gamma$ , in Combination with TGF- $\beta$ 1, on pro $\alpha$ 2(I) Collagen Promoter Activity in Human Skin Fibroblasts and NIH-3T3 Cells**

Cells	Control	TGF- $\beta$ 1	TGF- $\beta$ 1 + TNF- $\alpha$	TGF- $\beta$ 1 + IFN- $\gamma$	TGF- $\beta$ 1 + TNF- $\alpha$ + IFN- $\gamma$
HSF	3.25 $\pm$ 0.80 (1.00)	15.76 $\pm$ 3.62 (4.85)	4.96 $\pm$ 0.93 (1.53)	10.83 $\pm$ 3.09 (3.33)	0.97 $\pm$ 0.21 (0.30)
NIH-3T3	6.54 (1.00)	49.71 (7.60)	25.84 (3.95)	44.47 (6.80)	15.96 (2.44)

Cells were transfected with 20  $\mu$ g (human skin fibroblasts [HSF]) or 10  $\mu$ g (NIH-3T3 cells) of pro $\alpha$ 2(I) collagen/CAT plasmid, and the transfected cells were placed in DME + 1% FCS (HSF) or DME alone (NIH-3T3 cells). After a 3-h incubation, the growth factor(s) indicated were added, and the cells were incubated for an additional 40 h. The cells were harvested and the CAT activity was measured. The values are expressed as the percentage of conversion of [ $^{14}$ C]chloramphenicol to its acetylated derivatives per 10  $\mu$ g of cell extract protein; mean $\pm$ SEM of three separate experiments each performed with duplicate cultures (HSF), or mean of duplicates in a single experiment (NIH-3T3 cells). The values in parentheses indicate the change in CAT activity elicited by the growth factor(s) in relation to the corresponding untreated control cultures.

proximately to the same levels that were seen in control cultures (Table II). Interestingly, IFN- $\gamma$  (1,000 U/ml) together with TNF- $\alpha$  (10 ng/ml) did not further decrease the pro $\alpha$ 1(I) collagen mRNA levels in TGF- $\beta$ 1 treated cells, as compared to the effect noted with TNF- $\alpha$  alone (Table II). Again, incubation with growth factors did not change the transfection efficiency, as determined by the plasmid copy number (32).

In transient transfection experiments with cultured human skin fibroblasts, TNF- $\alpha$  (10 ng/ml) also suppressed TGF- $\beta$ 1-induced activation of pro $\alpha$ 2(I) collagen promoter activity nearly to the same level noted in untreated control cells (Fig. 3 and Table IV). These results indicated that TNF- $\alpha$  is a potent inhibitor of type I collagen gene expression and that it exerts its effects primarily at the transcriptional level. In support of this notion was the observation that co-treatment of transfected cells with TNF- $\alpha$  and IFN- $\gamma$  completely abolished the activation of pro $\alpha$ 2(I) collagen promoter by TGF- $\beta$ 1 in human skin fibroblasts (Fig. 3 and Table IV). Similar results were obtained in mouse NIH-3T3 cell cultures, although the suppression of pro $\alpha$ 2(I) promoter activity was less than that in human skin fibroblasts (Table IV). These results suggest synergistic inhibition of type I collagen gene expression by IFN- $\gamma$  and TNF- $\alpha$ .

**Effects of other growth factors on collagen gene expression.** Several additional growth factors, including EGF, PDGF, bFGF, and IGF-I, were also tested for their effects on pro $\alpha$ 2(I) collagen promoter activity. All four factors, tested in concentrations of 1, 10, or 100 ng/ml, were ineffective in modulating type I collagen promoter activity in NIH-3T3 cells using similar conditions under which TGF- $\beta$ 1 and TNF- $\alpha$  were clearly effective (results not shown). It is conceivable, therefore, that the changes previously observed as a result of exposure of connective tissue cells to these growth factors (see Discussion)

are not transcriptionally mediated, and may reflect alterations at posttranscriptional levels of gene expression. It is also conceivable that the major effects of these growth factors *in vivo* are related to the stimulation of fibroblast proliferation and/or migration.

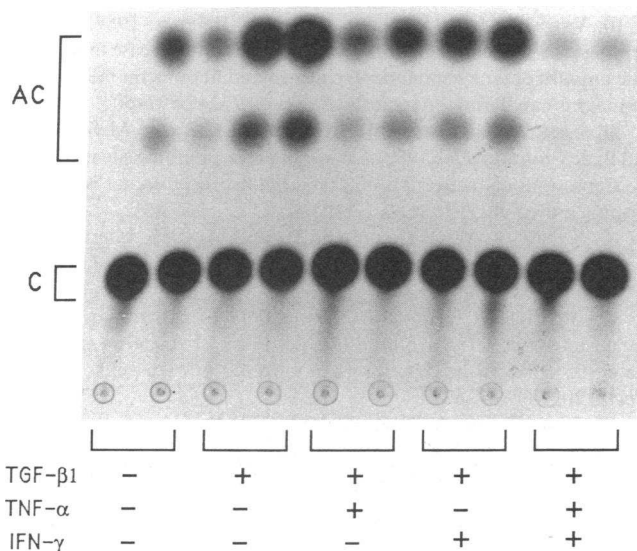
## Discussion

Collagen accumulation is the pathologic hallmark of several fibrotic diseases affecting a variety of organs. Excessive collagen deposition can lead to fibrosis of internal organs, as for example, in the case of pulmonary fibrosis or liver cirrhosis. Also, skin is often affected by fibrotic processes. The prototype of fibrotic diseases is progressive systemic sclerosis, a generalized connective tissue disorder that involves not only the skin but also the lungs, heart, kidneys, and the gastrointestinal tract. Several previous studies have indicated that activation of collagen biosynthetic pathway plays mechanistically a role leading to deposition of collagen. For example, collagen biosynthesis, as determined by the formation of radioactive hydroxyproline in fibroblast cultures established from these patients, is increased (19). Furthermore, type I procollagen mRNA levels are increased both in scleroderma cell cultures (33, 34), as well as in affected tissue *in vivo* as detected by *in situ* hybridizations (20, 35, 36), suggesting a regulation at the pretranslational level. Finally, recent studies utilizing nuclear run-on assays have suggested activation of collagen gene expression at the transcriptional level (37).

The precise mechanisms leading to activation of collagen gene expression in fibrotic diseases are currently unknown. However, recent observations that activation of collagen gene expression often occurs in the vicinity of inflammatory cells present in the lesional skin (20, 35, 36) suggest that polypeptide growth factors, such as cytokines elaborated by lymphocytes and monocytes, may enhance collagen gene expression in these diseases. In support of this hypothesis are observations indicating that TGF- $\beta$ 1 expression is markedly elevated in the lesional skin of patients with various forms of cutaneous fibrotic diseases (36). Since TGF- $\beta$  has been shown to be a potent upregulator of collagen gene expression in a variety of experimental situations, it may also play a role in the development of fibrotic disorders.

Two lymphokine factors, TNF- $\alpha$  and IFN- $\gamma$ , have been shown to suppress collagen gene expression in cell cultures (15, 16). Also, IFN- $\gamma$  has been shown to reduce collagen accumulation in experimental fibrosis in animals (38). On the basis of these observations, it has been suggested that interferons might potentially serve as a therapeutic modality to counteract collagen accumulation in fibrotic diseases (39).

In this study, we have examined the modulation of type I procollagen promoter activity by a variety of polypeptide growth factors, including TGF- $\beta$ 1, TNF- $\alpha$ , and IFN- $\gamma$ . This approach was made feasible by previous studies that established that the human pro $\alpha$ 2(I) collagen/CAT reporter gene chimeric construct used in our study is able to mimic physiological expression of the gene when transiently expressed in human fibroblastic cells (23). This construct contains ~ 3.5 kb of 5' flanking DNA from human pro $\alpha$ 2(I) collagen gene, cloned in front of the structural gene for CAT. Incubation of cells transfected with this chimeric construct with TGF- $\beta$ 1 resulted in marked up-regulation of the promoter activity both in cultured human skin fibroblasts and in mouse NIH-3T3



**Figure 3.** Assay of type I procollagen promoter activity in human skin fibroblast cultures incubated with TGF- $\beta$ 1 (5 ng/ml), TNF- $\alpha$  (10 ng/ml), and/or IFN- $\gamma$  (1,000 U/ml). The cell cultures were transfected with the pro $\alpha$ 2(I) collagen promoter/CAT gene construct pMS-3.5 CAT, and the cells were incubated in medium containing 1% fetal calf serum in the presence of the polypeptide growth factors, as indicated. The CAT activity was determined after 40 h of incubation, as described in Methods and in Fig. 1.



cells. The increases in the latter cells were up to 23-fold, while the corresponding increase in human skin fibroblasts was approximately 5-fold. In human skin fibroblasts, the increase noted in the promoter activity was accompanied by similar increases in pro $\alpha$ 1(I) and pro $\alpha$ 2(I) collagen mRNA levels. Thus, the increase noted in the type I procollagen mRNA steady-state levels appeared to reflect primarily activation of transcription of the gene. These results are in agreement with those previously reported by Rossi et al. (11), utilizing a mouse pro $\alpha$ 2(I) collagen/CAT gene construct in NIH-3T3 cells. In addition to the transcriptional activation, Penttinen et al. (40) have demonstrated that stabilization of mouse pro $\alpha$ 1(I) collagen mRNA also plays a role in TGF- $\beta$ 1-induced enhancement of collagen gene expression. In the human skin fibroblast system utilized in our study, the principal effect appears to be on the transcriptional level of type I procollagen gene expression. This conclusion was supported, in part, by the observation that TGF- $\beta$ 1 did not alter the CAT activity in the cells transfected with pSV2CAT construct, indicating that TGF- $\beta$  had no effect on the stability of CAT mRNA or protein.

TNF- $\alpha$  and IFN- $\gamma$  were both shown to counteract the TGF- $\beta$ 1-induced enhancement of type I procollagen gene expression, as determined at the mRNA steady-state level. The results suggested, however, that the mechanism of inhibition was different: TNF- $\alpha$  appeared to affect type I procollagen gene expression on the transcriptional level, while IFN- $\gamma$  appeared to exert its effect primarily through destabilization of the mRNA. These conclusions were supported by the observation that TNF- $\alpha$  had no effect on CAT activity in cells transfected with pSV2CAT, suggesting that neither the SV40 promoter nor CAT mRNA is susceptible to modulation by this polypeptide factor. However, IFN- $\gamma$  reduced the CAT activity in these cells to about half of that noted in the controls suggesting generalized enhancement of mRNA turnover. It should be noted that the growth factor effects in NIH-3T3 cells were obtained in serum-free medium. Similar results were obtained in human skin fibroblasts even though these cultures were supplemented with 1% fetal calf serum, indicating that endogenous factors present in serum did not interfere with the growth factor effects. These observations are in agreement with previous studies which have used culture media with different concentrations of fetal calf serum, varying from 0 to 10% (8–11, 15, 16).

An interesting observation was the synergistic effect of TNF- $\alpha$  and IFN- $\gamma$  on pro $\alpha$ 2(I) collagen promoter activity (Fig. 3 and Table IV). A potential explanation for this synergism is provided by the previous finding that IFN- $\gamma$  can increase the number of TNF- $\alpha$  receptors in cultured cells, thus making these cells more susceptible to TNF- $\alpha$  modulation (41). These observations would suggest that a combination of polypeptide factors, such as TNF- $\alpha$  and IFN- $\gamma$ , would provide a potential approach for a more efficient pharmacologic suppression of collagen accumulation in fibrotic diseases than afforded by either one of the peptide factors alone.

## Acknowledgments

The authors thank May Wu for skillful technical assistance, and Charlene D. Aranda and Debra Pawlicki for expert secretarial help. Dr. David R. Olsen, Collagen Corporation, Palo Alto, CA, kindly provided TGF- $\beta$ 1.

This study was supported in part by grants GM-28833, AR-35297, AR-38648, HL-41104, and T32-AR-07561. Dr. Kähäri is supported

by a Dermatology Foundation fellowship and the Finnish Cultural Fund.

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