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Transforming growth factor β (TGFβ) causes a persistent increase in steady-state amounts of type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts

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It has been previously shown that transforming growth factor β (TGFβ) is capable of stimulating fibroblast collagen and fibronectin biosynthesis. The purpose of this study was to examine the mechanisms involved in TGFβ stimulation of fibroblast biosynthetic activity. Our results indicate that TGFβ causes a marked enhancement of the production of types I and III collagens and fibronectin by cultured normal human dermal fibroblasts. The rate of collagen production by fibroblasts exposed to TGFβ was 2–3-fold greater than that of control cells. These effects were associated with a 2–3-fold increase in the steady-state amounts of types I and III collagen mRNAs and a 5–8-fold increase in the amounts of fibronectin mRNAs as determined by dot-blot hybridization with specific cloned cDNA probes. In addition, the increased production of collagen and fibronectin and the increased amounts of their corresponding mRNAs remained elevated for at least 72 h after removal of TGFβ. These findings suggest that TGFβ may play a major role in the normal regulation of extracellular matrix production *in vivo* and may contribute to the development of pathological states of fibrosis.

INTRODUCTION

Under normal circumstances, an accurate balance is maintained between the amount of connective-tissue macromolecules synthesized by fibroblasts and the amount removed from tissues [1]. The rates of production of these macromolecules are intrinsically regulated by three general mechanisms: modulation of the steady-state amounts of their mRNAs; control of the rate of mRNA translation; and variation in the amount of intracellular degradation of newly synthesized molecules. In addition, extra-fibroblastic signals may also influence the rates of matrix macromolecule biosynthesis, presumably by modifying one or more of these intrinsic mechanisms. A number of soluble products from inflammatory cells and platelets have been shown to affect connective-tissue metabolism *in vitro* [2,3], and it has been suggested that these factors may play a role in the regulation of matrix synthesis during normal and pathological fibrogenesis.

Transforming growth factor β (TGFβ) is a 25 kDa protein that was originally found in virally transformed rodent cells, and subsequently was shown to be present in most non-neoplastic adult tissues (for review see [4]). TGFβ exerts both growth-enhancing and growth-inhibitory effects on various cell lines. Recent studies *in vivo* of the effect of TGFβ have demonstrated accelerated healing when partially purified TGFβ was added to wire-mesh wound chambers in rats [5], as well as enhanced fibrosis and angiogenesis when TGFβ was subcutaneously injected into mice [6]. Consistent with these observations, we and others have shown that purified TGFβ causes a dose-dependent stimulation of collagen production by a variety of cell lines in culture, including dermal, lung and gingival fibroblasts [7–9] as well as osteoblastic cells [10]. Enhanced synthesis of fibronectin and collagen by cultured rodent fibroblasts in response to TGFβ has also been reported [11]. The precise mechanism of these effects is not yet known.

Since TGFβ is present in high concentration in platelets [12], the earliest cellular elements present in injured tissues, it is considered that TGFβ may be physiologically targeted to influence wound healing. In addition, the demonstration of TGFβ expression in activated lymphocytes [13], the principal cellular elements of chronic inflammatory reactions, suggests that TGFβ may play an important role in the fibrotic response uniformly associated with this process.

In the present work, we extended our studies on the effects of TGFβ on matrix macromolecule biosynthesis by cultured normal human dermal fibroblasts [7], and examined the mechanisms of this interaction. We found that TGFβ caused a severalfold stimulation of the production of fibronectin and types I and III collagens by several cultured normal human fibroblast lines, and that this effect appeared to be mediated largely by a concomitant increase in the steady-state amounts of specific transcripts for these proteins. In addition, we found that the increased production of these molecules and their elevated mRNA amounts persisted for at least 72 h after removal of TGFβ from the cultures. These findings indicate that TGFβ may play a crucial role in the regulation of fibroblast connective-tissue production in normal and pathological fibrogenesis.

Abbreviations used: TGF, transforming growth factor; EGF, epidermal growth factor.

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MATERIALS AND METHODS

Source of growth factors

A preparation of TGFβ (over 95% pure) obtained from fresh pig platelets was supplied by Dr. R. Lucas, of R and D Systems (Minneapolis, MN, U.S.A.) and was kept at −20 °C in a solution of 0.1 M-HCl containing 5% (w/v) pure human serum albumin. Epidermal growth factor (EGF) was purchased from Collaborative Research (Lexington, MA, U.S.A.).

Fibroblast cultures and labelling conditions

Fibroblasts were obtained from skin biopsies from normal individuals as described previously [14]. Early-passage cells (less than passage 6) were cultured in Eagle's minimal essential medium supplemented with 10% (v/v) 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 ml of fresh media containing 500 pM-TGFβ, 5% fetal-calf serum and 50 μg of ascorbate/ml in Eagle's medium was added every 24 h for 3 days. After 12 h of the last addition of TGFβ, 4 μCi of [^{14}C]proline/ml and 100 μg of β-aminopropionitrile/ml were added, and the incubation was continued for 24 h.

Analyses of labelled proteins

After the 24 h of labelling, the incubations were stopped. The media and cell layers were removed and, to prevent collagenolytic and proteolytic activity during further processing, they were transferred to cold tubes containing a mixture of proteinase inhibitors to yield the following final concentrations: EDTA, 5 mM; phenylmethanesulphonyl fluoride, 0.2 M; *N*-ethylmaleimide, 5 mM: *p*-aminobenzamidine hydrochloride, 1 mM. The cell layers were mechanically suspended with a rubber policeman, to avoid the use of proteolytic enzymes that may cleave cell-associated proteins. Samples were taken for quantitative determination of DNA by a fluorescence assay [15]. The combined media and cell-layer samples were then dialysed exhaustively against running water to remove unincorporated radioactive precursors. Total incorporation of [^{14}C]proline was measured in a scintillation spectrophotometer. Samples of media and cell homogenates from cultures labelled with [^{14}C]proline were used for determination of their hydroxy[^{14}C]proline content. For this, samples were hydrolysed in 6 M-HCl at 110 °C for 18 h, and hydroxy[^{14}C]proline was assayed in the hydrolysates by a modified chemical procedure [16]. In selected experiments the amount of newly synthesized collagen was quantified by enzymic digestion with pure bacterial collagenase (Cooper Biomedical) by the method of Peterkofsky & Diegelmann [17].

Precipitation of fibronectin with antibodies

Immunoprecipitation of radioactive fibronectin synthesized by the fibroblasts was performed with commercially available purified antibodies to human plasma fibronectin (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.). Samples of labelled media were dialysed exhaustively against 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4 at 4 °C. The cell layers were first homogenized in 1.0 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4 at 4 °C, and then dialysed against the 0.15 M-NaCl/50 mM-Tris/HCl buffer. Insoluble material representing less than 5% of the radiolabelled proteins, was removed by centrifugation at 5000 *g* for 30 min. Two different concentrations of the antibodies were added to each sample, and the mixtures were incubated at room temperature for 16 h. The control samples did not contain any antibodies. Protein A (10 μg/ml) was added to facilitate precipitation of fibronectin–anti-fibronectin complexes. The samples were mixed and centrifuged for 5 min at 960 *g*, and samples of the supernatants and the pellets were taken for scintillation counting. Other samples were also processed for SDS/polyacrylamide-gel electrophoresis and fluorography as described below.

Gel electrophoresis

Electrophoresis of labelled proteins from the media of control cultures was performed on 5%- or 8%-polyacrylamide slab gels in SDS under reducing conditions. 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 as previously described [18].

Isolation and analysis of total RNA from skin fibroblasts

For this purpose, confluent fibroblasts were cultured in T 175 plastic flasks as described above in the presence or absence of TGFβ. At the end of the labelling period, the media were decanted and the cells washed twice with 5.0 ml of 0.02% Na_4EDTA. The cells were then scraped off their substrate and washed with 10 ml of 0.02% Na_4EDTA. The combined washes were then centrifuged at 600 *g* for 5 min. The cell pellets were resuspended by vortex-mixing in 5 vol. of 4 M-guanidinium isothiocyanate/5 mM-sodium citrate/0.1 M-β-mercaptoethanol/0.5% Sarkosyl. A portion (0.1 vol.) was removed for DNA determination. Total fibroblast RNA was then isolated by the CsCl-discontinuous-gradient method outlined by Maniatis *et al.* [19]. For dot-blot analysis, total RNA was dissolved in 10 × standard saline citrate ('SSC') buffer (0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0) containing 9% (v/v) formaldehyde, and the solutions were heated to 65 °C for 3 min and then quick-chilled in an ice bath. Portions of each RNA sample, containing 0.25, 0.5 and 1.0 μg of RNA, were blotted on to a nylon filter with a Schleicher and Schull dot-blot apparatus as described by Kafotos *et al.* [20]. Hybridization to the filter-bound nucleic acid was performed as previously described [21]. Each of the RNA samples was analysed at each of the three concentrations in duplicate. Autoradiograms of RNA dot-blots were analysed with a densitometer, and the areas under the obtained peaks were quantified by using a planimeter. The areas under the peaks were linear with respect to the quantities of total RNA hybridized.

The cDNA probes Hf677 and RJ5, which are specific for α1(I) and α1(III) human collagen genes respectively, used in this analysis have been previously isolated and shown to be chain-specific under the conditions of hybridization and washing employed here [21].

The cDNA probe pFH1 specific for fibronectin was kindly supplied to us by Dr. Francisco Baralle [22].

RESULTS

Effects of TGFβ on protein and collagen synthesis by confluent normal human fibroblasts

The effects of TGFβ on [^{14}C]proline incorporation and hydroxy[^{14}C]proline and fibronectin synthesis by confluent fibroblast cultures derived from four normal individuals are shown in Table 1. The concentration of TGFβ employed was shown to cause optimal stimulation of collagen synthesis in normal human dermal fibroblasts in a previous study [7]. The incorporation of [^{14}C]proline into cell and media macromolecules was stimulated from 87 to 187% over control values by TGFβ. A parallel increase in the amount of hydroxy[^{14}C]proline synthesis by the cultures was also seen. In selected experiments, the amount of newly synthesized collagen in control or TGFβ-treated cultures was determined by quantifying the collagenase-sensitive radiolabelled protein from the media. TGFβ caused a quantitatively similar increase in newly synthesized collagenase-sensitive protein (results not shown), confirming that the increase in hydroxy-[^{14}C]proline in TGFβ-treated cultures was not due to overhydroxylation of incorporated prolyl residues. In the present studies we found that daily addition of fresh TGFβ was necessary for optimal stimulation of collagen and protein biosynthesis, since the results obtained with only one addition on day 1, followed by labelling 72 h later as described previously [7], were occasionally inconsistent.

Effects of EGF alone or in combination with TGFβ on fibroblast proliferation and collagen synthesis

The transformation of normal rat kidney fibroblasts to anchorage-independent colony formation requires, in addition to TGFβ, the structurally and antigenically distinct EGF [23]. In order to investigate whether this synergism also plays a role in the stimulation of proliferation and collagen production by human dermal fibroblasts, we exposed confluent cultures to optimal concentrations of TGFβ, EGF and the combination of both factors. When proliferation of confluent fibroblast cultures was determined, we found that TGFβ did not cause significant cell growth, as determined by DNA assay and cell counts. EGF stimulated proliferation by approx. 36% as compared with untreated cultures. Incubation of cultures with a combination of EGF and TGFβ resulted in 29% increase in DNA compared with controls, suggesting that no synergistic effect on proliferation occurred. We next examined the effect of these two factors on collagen production. We found that TGFβ caused a 3-fold increase, whereas EGF inhibited collagen production, when compared with untreated cultures. Furthermore, addition of EGF to TGFβ-treated cultures resulted in partial abrogation of the collagen-stimulatory effect seen when TGFβ alone was used.

Characterization of labelled proteins

In order to characterize the labelled macromolecules synthesized by control or TGFβ-treated cultures, the media and cell layers were examined by SDS/polyacrylamide-slab-gel electrophoresis under reducing conditions and subsequent fluorography. As shown in a representative fluorograph (Fig. 1), treatment of cultures with TGFβ resulted in changes in the intensity of several electrophoretic bands. When equal-volume portions of media (100 μl) were examined, there was an increase in the relative intensity of the bands migrating in the regions of fibronectin, intact procollagens, pC collagens and α1 and α2 collagen chains. Similar analysis of cell layers showed a marked increase in the intensity of α1 and α2 chains.

The distribution of radioactivity in the different bands was calculated by measurement of the areas under the individual peaks. These results demonstrate a significant increase in the area under peaks representing newly synthesized fibronectin, and all collagen products in the TGFβ-treated cultures compared with the control cultures. Furthermore, an increase in the ratio of partially and fully processed collagen to intact procollagen was also seen under the influence of TGFβ as described previously [7].

To measure quantitatively the types of collagen present in the control and TGFβ-treated cultures, the ratios of labelled α1 to α2 chains were determined by SDS/polyacrylamide-gel electrophoresis under reducing conditions after enzymic digestion with pepsin to convert collagen precursors into their respective α-chains. The molar ratios of the α1 to α2 chains calculated from planimetry measurements of the fluorographs were 2.9:1 in the controls and 2.8:1 in the TGFβ-treated

Table 1. Effect of TGFβ on total protein, collagen and fibronectin production by confluent normal dermal fibroblasts

Confluent cultures of human skin fibroblasts were incubated with control medium or with medium containing 500 pM-TGFβ for 48 h and then labelled with [^{14}C]proline for 24 h, except for cell line N10, which was labelled for only 6 h. Labelled proteins in the media and cell layers were assayed for [^{14}C]proline incorporation and hydroxy[^{14}C]proline content. Fibronectin was determined by measurement of radioactivity precipitated by antibody to fibronectin (see the Materials and methods section). The values shown represent the averages of two separate experiments each performed in triplicate.

	Total [^{14}C]proline			Hydroxy[^{14}C]proline			Fibronectin		
	10^{-3} × Radioactivity (c.p.m.)		(% change)	10^{-3} × Radioactivity (c.p.m.)		(% change)	10^{-3} × Radioactivity (c.p.m.)		(% change)
Cell line	Control	TGFβ		Control	TGFβ		Control	TGFβ	
N7	35.6	102.1	+187	4.4	10.8	+145	12.0	69.4	+478
N8	40.7	76.0	+87	6.0	9.7	+62	22.3	62.0	+178
N9	46.6	107.7	+131	5.5	15.1	+174	13.2	63.3	+380
N10	10.2	23.0	+125	1.2	2.7	+118	–	–	–

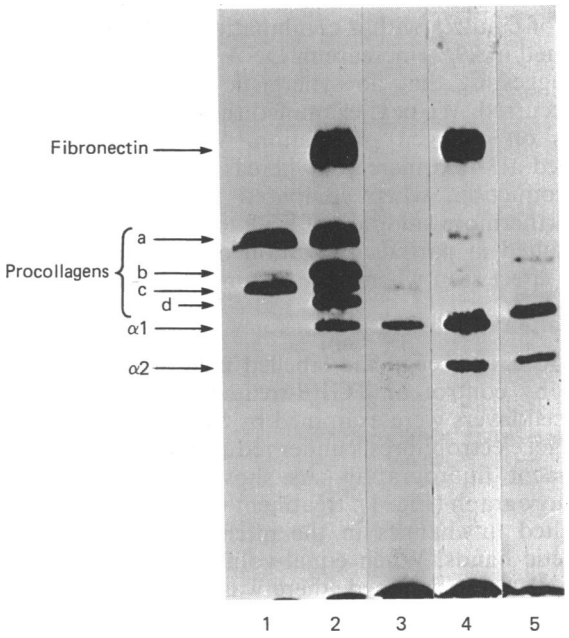

Fig. 1. Fluorograph of SDS/polyacrylamide-slab-gel electrophoresis of labelled macromolecules from medium and cell layer of untreated and TGFβ-treated fibroblast cultures

Confluent cultures of normal dermal fibroblasts were incubated in the presence or absence of TGFβ and labelled with [^{14}C]proline. After 24 h of labelling, cells and media were harvested, and equal samples were processed for electrophoresis and fluorography as described in the Materials and methods section. Lane 1: medium from untreated cultures. Lane 2: medium from TGFβ-treated cultures. Lane 3: cell layer from untreated cultures. Lane 4: cell layer from TGFβ-treated cultures. Lane 5: standard type I procollagen and collagen chains. Arrows: a, pro-α1; b, pC-α1; c, pro-α2; d, pC-α2.

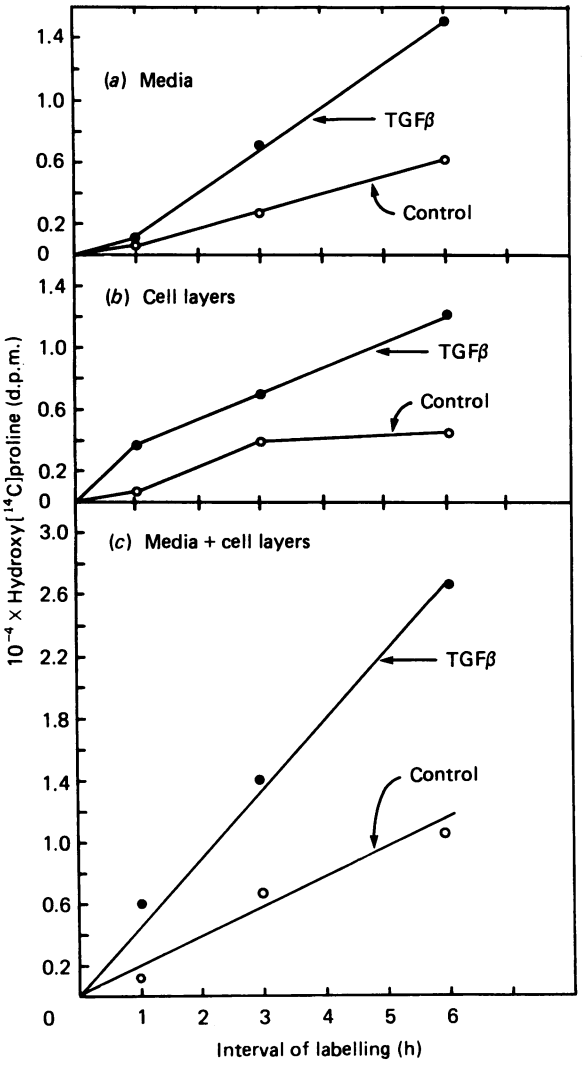

Fig. 2. Short-term kinetics of collagen synthesis and secretion in control and TGFβ-treated fibroblast cultures

Confluent cultures of normal human dermal fibroblasts were incubated under control conditions or with TGFβ for 16 h and then labelled with [^{14}C]proline for various periods. Triplicate wells were harvested 1, 3 and 6 h after labelling, and the amount of hydroxy[^{14}C]proline present in the media and cell layers was determined as described in the Materials and methods section. (*a*) Media; (*b*) cell layers; (*c*) total (cell layers + media). Each value represents the average of triplicates: S.E.M. was less than 10% for each triplicate set.

cultures. These findings indicate that the proportion of newly synthesized type I and type III collagens did not change after TGFβ treatment. These findings were confirmed by discontinuous electrophoresis (results not shown).

To quantify the increase in fibronectin production in TGFβ-treated cultures, specific immunoprecipitation with antibodies to human plasma fibronectin was used. The precipitates contained only a 225 kDa non-collagenous protein, which was identified as fibronectin by comparison with the electrophoretic mobility of authentic fibronectin standards, whereas the supernatants contained a mixture of procollagen and collagen molecules. Measurement of the radioactivity precipitated by the antibodies to fibronectin showed that TGFβ caused a marked increase in [^{14}C]proline incorporation into fibronectin as compared with controls (Table 1).

Effects of TGFβ on the kinetics of fibroblast collagen production

To characterize further the stimulatory effects of TGFβ on collagen production, we examined the kinetics of fibroblast collagen production after exposure to the growth factor. For this purpose, cultures were incubated in the presence or in the absence of TGFβ for 16 h and then were labelled with [^{14}C]proline for periods of 1, 3 and 6 h, and the amount of hydroxy[^{14}C]proline present in the culture media and cell layers was determined at each time interval. The results shown in Fig. 2 demonstrate that there was a progressive increase in total (cell layers + media) hydroxy[^{14}C]proline production with time in control and TGFβ-treated cultures. The rate of hydroxy[^{14}C]proline production, however, was markedly higher at each pulse period in the TGFβ-treated cultures compared with controls. For example, the increase in hydroxy[^{14}C]proline between 3 h and 6 h in control cultures was approx. 40.3%, whereas in the TGFβ-treated cells it was 99.8%. This difference in the kinetic pattern of hydroxy[^{14}C]proline production between control and TGFβ-treated cultures resulted in large

differences in total hydroxy[^{14}C]proline accumulation, and after 6 h of labelling the TGFβ-treated cells produced 150% more hydroxy[^{14}C]proline than did controls.

Persistence of elevated collagen and fibronectin synthesis after removal of TGFβ

In order to determine if the stimulatory effect of TGFβ required its continued presence in the media, cultures were exposed to TGFβ for 24 h and then the media were removed and replaced with fresh media which did not contain TGFβ. Collagen production was assayed by labelling triplicate cultures for 24 h periods with [^{14}C]-proline at 24, 48 and 72 h after the removal of TGFβ. We found that cultures labelled during the 24 h of exposure to TGFβ showed a 2-fold stimulation of collagen production in both media and cell layers. The stimulation of collagen production persisted for at least 72 h after removal of TGFβ (Fig. 3). In fact, the amount of hydroxy[^{14}C]proline synthesized from 24 to 48 h after removal of TGFβ (period 48–72 h in Fig. 3) was 6-fold higher than that synthesized in control cultures, and the amount synthesized in the subsequent 48 to 72 h (period 72–96 h in Fig. 3) was still 5-fold greater than in the untreated cells. The enhanced rate of fibronectin production also persisted, so that, in samples labelled from 48 to 72 h after removal of the growth factor, the amount of newly synthesized fibronectin was approx. 2–3-fold higher than in the untreated cultures.

Steady-state amounts of collagen and fibronectin mRNAs in control and TGFβ-treated fibroblasts

The steady-state amounts of mRNAs for types I and III collagens and fibronectin in control and TGFβ-treated cultures were analysed by dot-blot hybridization, by using specific cDNA probes for these molecules (Fig. 4). The fluorographs of the dot-blots were quantified by densitometric analysis. The results shown in Table 2 indicate that RNA from TGFβ-treated fibroblasts displayed a substantial increase in types I and III collagen and fibronectin-specific transcripts as compared with equal amounts of RNA from control fibroblasts.

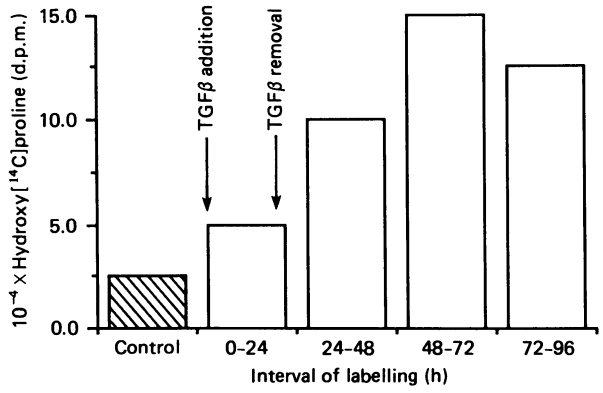

Fig. 3. Persistence of stimulation of fibroblast collagen synthesis after removal of TGFβ from cultures

Confluent cultures of normal human dermal fibroblasts were incubated with TGFβ for 24 h, and then the media were replaced with fresh media without TGFβ. The cultures were labelled with [^{14}C]proline for 24 h during exposure to TGFβ and at various intervals after removal of TGFβ from the media and the amount of hydroxy[^{14}C]-proline synthesized during this period was determined (see the Materials and methods section). Each value represents the average of two experiments.

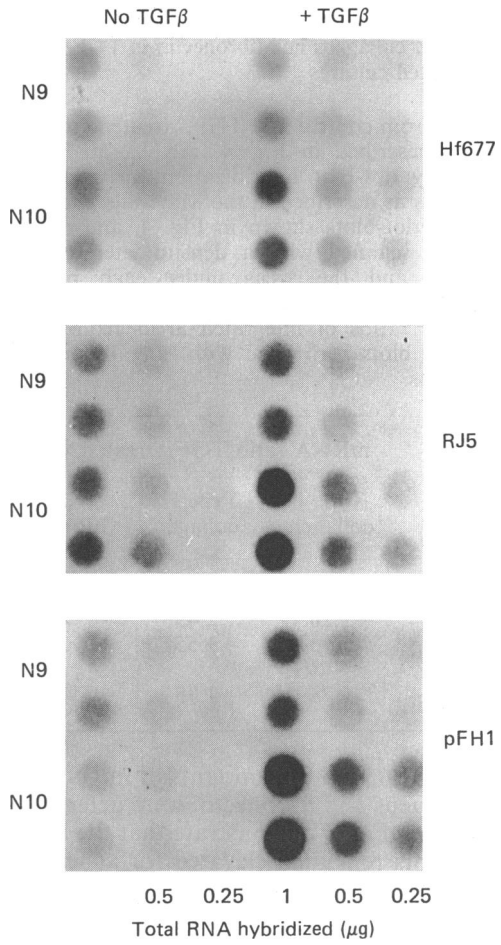

Fig. 4. Dot-blot hybridization with human cDNA probes specific for α1(I) and α1(III) collagen chains and fibronectin of RNA isolated from untreated and TGFβ-treated normal fibroblasts

Total RNA was isolated by guanidinium isothiocyanate extraction followed by CsCl-discontinuous-density-gradient centrifugation as described in the Materials and methods section. Duplicate samples containing 0.25, 0.5 and 1.0 μg of RNA from untreated and TGFβ-treated cultures were bound to nylon filters, and dot-hybridized to one of three specific ^{32}P-labelled cDNA probes. Cloned cDNAs were as follows: human α1(I) collagen, Hf677; α1(III) collagen, RJ5; fibronectin, pFH1. After hybridization and washing, the filters were subjected to autoradiography and then scanned with a linear-drive densitometer.

Steady-state amounts of collagen and fibronectin mRNAs after removal of TGFβ

To determine if the persistence of increased biosynthesis of collagen and fibronectin observed in cultures exposed to TGFβ after removal of the growth factor was correlated with a persistent increase in their respective mRNAs, dot-blot hybridization was performed in cultures exposed to TGFβ for 24 h at various intervals after its removal. Cultures were killed 48 and 72 h after

Table 2. Ratio of steady-state amounts of mRNAs for type I and type III collagens and fibronectin in TGFβ-treated and untreated cultures

Total RNA from control and TGFβ-treated cultures was isolated as described in Fig. 4. mRNA analyses were performed by dot-blot hybridization by using specific cDNA probes as described in the Materials and methods section. The dot-blots shown in Fig. 4, and others not shown, were scanned with a densitometer after autoradiography, and the areas under each peak were determined by using a planimeter. The values shown represent the ratios of integrated areas from duplicate hybridization blots performed with three different RNA concentrations.

	mRNA ratio TGFβ-treated/control		
Cell line	Type I collagen	Type III collagen	Fibronectin
Expt. 1			
N10	2.26	2.45	8.16
N9	1.36	1.55	2.26
Expt. 2			
N9	1.78	1.35	2.38

removal of TGFβ, and the amounts of mRNA for types I and III collagens and fibronectin were determined. The results shown in Fig. 5 demonstrate that the amounts of these transcripts remained elevated for at least 72 h. A remarkable observation was that the amounts of mRNA for types I and III collagens continued to increase, so that at 72 h they were respectively 92% and 102% higher than those obtained immediately after exposure to TGFβ. The amounts of fibronectin mRNA showed a slight decline at 48 and 72 h after removal of TGFβ, but were 82 and 91% higher than the amounts in untreated cells respectively.

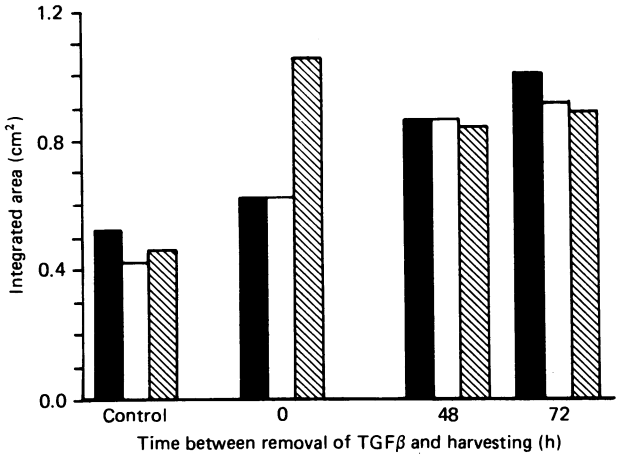

Fig. 5. Persistence of elevated steady-state amounts of mRNA for α1(I) and α1(III) collagen chains and fibronectin after removal of TGFβ

Confluent cultures of normal human dermal fibroblasts were incubated with TGFβ for 24 h, and the media were then replaced with fresh media without TGFβ. At the end of exposure to TGFβ and at various times after the removal of TGFβ from the cultures, cell layers were harvested and total RNA was isolated and hybridized with specific cDNA probes as described in the Materials and methods section. After hybridization, the nylon filters were subjected to autoradiography, and scanned. Areas under peaks were determined by planimetry. Control cultures were not exposed to TGFβ. mRNA: ■, type I collagen; □, type III collagen; ▨, fibronectin.

DISCUSSION

The results presented here demonstrate that TGFβ causes enhancement of the production of collagen and fibronectin, two important biosynthetic products of normal human dermal fibroblasts. These effects were observed in all four normal fibroblast cell lines examined. The increased production of collagen was confirmed by determining [^{14}C]proline incorporation into collagenase-sensitive proteins. The stimulation of collagen and fibronectin production was independent of cell proliferation, as TGFβ did not cause an increase in cell counts or DNA content.

A number of biological effects induced by TGFβ *in vitro* appear to require the simultaneous presence of EGF in culture. For example, the concerted action of both factors is necessary for the induction of anchorage-independent growth of NRK fibroblasts [23]. We have found that addition of EGF was necessary to induce proliferation of quiescent dermal fibroblasts, and that TGFβ alone had no mitogenic effect. Furthermore, our results indicate that EGF antagonized the TGFβ-induced stimulation of collagen production by these cells. Fine & Goldstein [8] reported that treatment of quiescent lung fibroblast cultures with a combination of TGFβ and EGF resulted in additional increase in collagen formation. This apparent discrepancy may be due to the proliferative effect of EGF on fibroblasts; when the data of Fine & Goldstein [8] are corrected for the increased cell numbers in EGF-treated cultures, EGF appears to abrogate the TGFβ-induced stimulation of fibroblast biosynthesis.

Examination of the relative amounts of type I and type III collagens in the media indicated that both were proportionately increased in TGFβ-treated cultures. Similar results were described in lung fibroblasts exposed to TGFβ [8]. The finding of enhanced fibronectin production accompanied by an increase in newly synthesized collagen raises the possibility that excess fibronectin may interfere with the normal extracellular breakdown of collagen by collagenase [24], thus resulting in its net accumulation. However, it has been demonstrated that TGFβ causes stimulation of collagenase activity in cultured human fibroblasts [25]. Furthermore, our findings in short-term labelling experiments, that increased collagen accumulation occurred within 60 min of initiation of labelling, indicate that the TGFβ effects are not due to decreased extracellular degradation of the newly synthesized collagens.

We also found that TGFβ altered the kinetics of fibroblast collagen production. Cells that had been exposed to TGFβ displayed a 2–3-fold higher rate of production of radiolabelled collagen compared with control cells. This observation suggests that TGFβ may interfere with normal regulatory mechanisms in these cells. Similar alterations in the kinetics of collagen production have been reported in fibroblasts from patients with scleroderma, a disease characterized by excessive tissue collagen accumulation [26]. These results

are more remarkable since TGFβ also increased the processing of procollagen to collagen and the accumulation of fully processed α-chains in the cell layers. The findings raise questions regarding the role of procollagen peptides in the physiological regulation of collagen synthesis. The accelerated processing of procollagen chains, coupled with its increased production, would be expected to result in the accumulation of large amounts of procollagen peptides in the immediate pericellular environment. The continued production of increased amounts of procollagen under these conditions suggests either that these peptides do not exert a regulatory effect in intact cells or that their effects are abolished by TGFβ.

To investigate the mechanism of stimulation of collagen and fibronectin production, we determined the amounts of types I and III collagen- and fibronectin-specific mRNA transcripts in control and TGFβ-treated fibroblasts. We found that TGFβ-treated cells demonstrated a consistent increase in each of these mRNAs as compared with untreated fibroblasts. A possible explanation for these findings is that TGFβ-treated fibroblasts display increased transcription of collagen and fibronectin genes; in most experimental situations the production of these proteins is regulated at a transcriptional level [27]. However, there are other possible mechanisms that may account for the higher amounts of mRNAs found in these cells, such as increased mRNA stability, or a combination of increased transcription and decreased degradation. A combination of these two factors has been shown to be responsible for the increased collagen production demonstrated in avian fibroblasts exposed to ascorbic acid [28]. In addition, other mechanisms may play a role in mediating the effects of TGFβ. For example, in embryonic rat osteoblasts treated with TGFβ, no precise correlation between increased amounts of mRNA for α1 chain of type I collagen and the expression of the gene product was found [10]. These results suggest that both transcriptional and translational regulation by TGFβ may be operative in determining its effects on the rate of collagen production. From our experiments, it is not clear whether the increases in collagen and fibronectin mRNAs found in TGFβ-treated fibroblasts are due to accelerated transcription or to increased stability of their corresponding mRNA, and studies are now needed to examine these questions. Further studies will also be necessary to determine whether TGFβ may also exert translational control of collagen production by causing enhanced net collagen-mRNA translation or by interfering with the feedback inhibition of translation.

One remarkable finding of the present studies was the demonstration that TGFβ-induced stimulation of collagen and fibronectin production, as well as the increase in their specific mRNAs, persisted for at least 72 h after the removal of the TGFβ from the cultures. These data suggest that TGFβ induces a persistent alteration in the regulation of fibroblast collagen and fibronectin biosynthesis. It can be speculated that the continued increase in the rate of collagen production by cultures even after removal of TGFβ results from increased stability of the newly transcribed collagen and fibronectin mRNAs. This effect could be due to induction or stimulation of synthesis of a protein or proteins capable of a decay-protective effect for the newly synthesized transcripts [29].

The results presented here suggest that TGFβ may be an important physiological regulator of the normal maintenance of extracellular matrix and tissue repair. Furthermore, it may be speculated that in pathological fibrosis, where accumulation or activation of inflammatory cells and platelets is suspected to play a pathogenetic role, TGFβ, a product of both of these cells, may be a crucial signal for the development of fibrosis.

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