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PERSISTENT EFFECT OF TGF-β_1 ON EXTRACELLULAR MATRIX GENE EXPRESSION IN HUMAN DERMAL FIBROBLASTS

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TGF-β, a potent inducer of the extracellular matrix, is also known to stimulate its own synthesis. In this report we have analyzed long term effects of TGF-β_1 on its own expression and on the expression of extracellular matrix genes. We demonstrated that 24 hours of incubation of human dermal fibroblasts with TGF-β_1 (1 ng/ml) under serum free conditions resulted in an elevated expression of TGF-β_1 , collagen α2(I) and fibronectin mRNAs that persisted at least 96 hours after removal of TGF-β_1. These data suggest the possibility of persistent in vivo activation of target cells following exposure to TGF-β_1.

TGF-β is a multifunctional member of a family of polypeptide growth factors that regulate cell growth and differentiation. The polypeptide originally described as TGF-β consist of a homodimer of β_1 subunits each with a molecular weight of 12.5 kd (1). Most of the TGF-β secreted by platelets or cultured cells exists in a latent form that consists of mature TGF-β (25 kd) noncovalently associated with sequences from the remainder of the precursor (74 kd) and a third unidentified component (135 kd) (2). Since almost all cells produce TGF-β and its receptor appears to be universally and constitutively expressed, activation of the latent form is believed to be a regulatory step in controlling its physiological action. Active TGF-β has been found in supernatants derived from cultured dermal fibroblasts (3). TGF-β has potent effects on the formation of extracellular matrix, stimulating synthesis of several matrix components, including collagens type I, III, and V and fibronectin (4). Regulation of matrix gene expression appears to involve both transcriptional and postranscriptional levels (5,6). In addition, TGF-β inhibits matrix degradation by increased synthesis of inhibitors of proteases involved in matrix degradation and by decreased synthesis of the proteases themselves (4).

TGF-β also increases steady-state levels of its own message in a variety of normal and transformed cells in culture (7). Two distinct regions in the TGF-β promoter and several yet uncharacterized nuclear factors associated with autostimulation have been identified (8).

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TGF-β has been associated with fibrosis both in vivo and in vitro and may play a role in the pathogenesis of scleroderma (9). The colocalization of TGF-β_2 around cells expressing collagen mRNA was demonstrated by in situ studies in early scleroderma lesions (10), while TGF-β_1 was shown to be expressed at a detectable levels in the affected tissues from patients with morphea and diffuse fascitis (11). We wished to determine whether TGF-β1 could induce in vitro the phenotype consistent with that of scleroderma fibroblasts (12). In this report we explore possible long term effects of TGF-β on its own message and on the induction of extracellular matrix genes in cultured human dermal fibroblasts.

Materials and Methods

Cells-Human adult skin fibroblasts were obtained by biopsy of the dorsal forearm skin of healthy adult human volunteers following informed consent and institutional approval. Primary explant cultures were established in 25 cm^2 culture flasks (Costar, Cambridge, MA) in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2 mM L-Glutamine, 50 μg/ml gentamicin sulfate (Quality Biological, Inc., Gaithersburg, MD) and 5 μg/ml amphotericin (Gibco, Grand Island, NY) the latter for one week. Monolayer cultures were maintained at 37°C in a humidified atmosphere of 10% CO_2/90% air and fed with DMEM containing 10% FCS, 2 mM L-glutamine and 50 μg/ml gentamicin.

RNA isolation and Northern blot analysis-Cells were grown to confluence in DMEM supplemented with 10% FCS in 15 cm-diameter dishes (Falcon Products, Cockeysville, MD), washed three times with DMEM/0.1% BSA followed by a 24-hour preincubation in DMEM/0.1% BSA. Medium was then replaced with serum-free medium containing 1 ng/μl of TGF-β_1 (R&D Systems, Minneapolis, MN) and, after varying times, total RNA was extracted using an acid guanidinium thiocyanate-phenol-chloroform method (13). Ten μg of total RNA was electrophoresed on 1% agarose/formaldehyde gel and blotted onto nylon filters (ICN Biochemicals, Inc., Cleveland, OH). The filters were baked, prehybridized, and hybridized as follows: after a 2 hour incubation at 42°C in prehybridization buffer [50% deionized formamide, 5X Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 5X SSPE and 100 μg/ml herring sperm DNA], filters were hybridized overnight at 42°C with 5 x 10^6 cpm/ml of labelled probe at 42°C in the same buffer but without herring sperm DNA. Blots were washed twice in 2X SSPE for 15 min at room temperature, twice in 2 X SSPE/2% SDS for 45 minutes at 55°C and twice in 0.1 x SSPE for 15 min at room temperature. Filters were exposed to Kodak X Omat AR film at -70°C overnight. Probes used [TGF-β_1-220 bp PvuII fragment, derived from cDNA; fibronectin-380 bp PstI fragment corresponding to cell binding domain; and collagen α2(I)-4 kb EcoRI fragment corresponding to 3'region of the gene; GAPDH - plasmid containing rat cDNA] were labelled with a random primed DNA labelling kit (Boehringer Mannheim, Indianapolis, IN). RNA was quantitated by densitometric scanning of the autoradiograms.

[^{125}I]-labeled TGF-β_1 assays-Cells were plated into 24-well plates in DMEM supplemented with 10% FCS and grown to confluence, then incubated in DMEM/0.1% BSA for 24 hours. [^{125}I]-labeled TGF-β_1 (sp. act.-240 μCi/μg) (R&D System, Minneapolis, MN) was added at a concentration of 1ng/ml in 0.3 ml of DMEM/0.1%BSA for 24 hours. For each well medium was collected and the well was washed twice with fresh medium which also was collected. This procedure of medium change and collection was repeated every 24 hours for 96 hours. At each time point, cells from two parallel wells were

solubilized by 0.1%NaOH/0.1% SDS and collected. Extracellular matrix attached to the bottom of the well was collected separately by scraping. Amount of [^{125}I] associated with medium, cell layer and matrix in each time point was determined by gamma scintillation techniques.

Results

We have examined effects of TGF-β_1 on its own message in human dermal fibroblasts and have reproduced results of others (with other cell lines) (7) showing that TGF-β_1 is autostimulatory. We then examined how long the elevated level of TGF-β_1 mRNA persists after TGF-β_1 stimulation both in the presence and absence of TGF-β_1. To eliminate the effect of other serum-derived factors, including variable and unknown concentrations of TGF-β, experiments were conducted in serum-free conditions.

In a representative experiment cells were grown to confluence in DMEM containing 10% FCS then synchronized in DMEM/0.1% BSA for 24 hours. In one set of dishes, TGF-β_1 (1ng/ml) was added for 24 hours. Following this treatment, residual TGF-β_1 was removed by extensive washes and cells were incubated in DMEM/0.1% BSA for 96 hours with serum-free medium change every 24 hours. A second set of dishes was treated in a similar fashion with the exception that fresh TGF-β_1 (1ng/ml) was added with every 24 hour medium change. As shown in Fig.1, steady state levels of TGF-β_1 mRNA were elevated for at least 96 hours in both conditions.

TGF-β has been shown to adhere nonspecifically to various surfaces. In order to insure that exogenous TGF-β_1 has been completely washed out and is not present to stimulate its own message, we used [^{125}I]-labeled TGF-β_1 under the same experimental conditions. We then measured levels of [^{125}I] in the medium and in association with extracellular matrix and cell layer. Medium change after 24 hours was sufficient to remove [^{125}I] almost completely from the medium or extracellular matrix, although a constant small amount of [^{125}I] remained associated with cell layer (2%) (table I). As shown by Frolik et al (14) TGF-β_1 is rapidly degraded by lysosomal enzymes and therefore, it is likely that this latter radioactivity represents TGF-β_1 degradation products accumulated within the cell. The other possibility is that this radioactivity represents TGF-β_1 stably bound

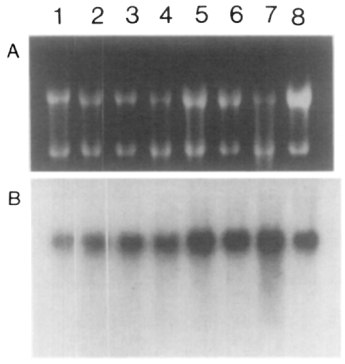

Fig. 1. Time course of TGF-β_1 effects on its mRNA levels. Northern blot analysis of total RNA isolated from cells incubated for 24 hours in DMEM/0.1% BSA (lane 1), stimulated with TGF-β_1 (1 ng/ml) for 24 hours (lane 2) and restimulated with TGF-β every 24 hours for 48 hours (lane 3), 72 hours (lane 5) and 96 hours (lane 7); or from cells after 24 hour stimulation with TGF-β, following incubation in DMEM/0.1% BSA for 48 hours (lane 4), 72 hours (lane 6) and 96 hours (lane 8). Panel A represents ethidium bromide staining of the gel before transfer, panel B, 2.4 kb TGF-β mRNA.

to one of the cell membrane components (15).

We then conducted experiments to determine if the expression of TGF-β inducible genes, such as collagen and fibronectin, also persist after exogenous TGF-β_1 removal. As depicted in Fig. 2 elevated steady-state levels of collagen and fibronectin mRNAs are present 4 days after removal of TGF- β_1.

Quantitation of mRNA levels normalized against 28S and 18S RNAs revealed 2.8 fold increase of procollagen α2(I) mRNA and 3.6 fold increase

Table I

Amount of ^{125}I(cpm)[1]

	Medium	Cell layer	Matrix
Collection time			
24h	101353 (85)[2]	5184 (4.35)	180.5 (0.15)
48h	2087 (1.7)	2644 (2.2)	125 (0.1)
72h	856 (0.7)	2654 (2.2)	244 (0.2)
96h	623 (0.5)	2010 (1.6)	222 (0.18)

[1] Total radioactivity added = 119 118 cpm.

[2] Numbers in parentheses represent % of total radioactivity. Experiment was repeated three times and values from one representative experiment are shown.

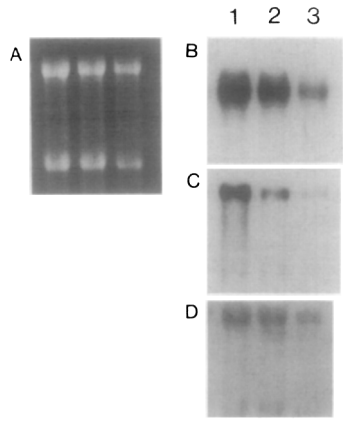

Fig. 2. TGF-β_1 effects on collagen and fibronectin mRNA levels. Cells were incubated for 24 hours in DMEM/0.1% BSA, then stimulated with TGF-β (1 ng/ml) for 24 hours (lane 1). TGF-β_1 was washed off and cells were incubated with DMEM/0.1% BSA for 96 hours with medium change every 24 hours (lane 2). Control cells were incubated only with DMEM/0.1% BSA with every 24 hour medium change for equal time period (lane 3). Panel A, ethidium bromide staining of the gel before transfer, panel B, 5.7 kb collagen α2(I) mRNA, panel C, 9.1 kb fibronectin mRNA, panel D, 1.4 kb GAPDH mRNA.

of fibronectin mRNA levels 96 hours after removal of TGF-β_1 as compared to mRNA levels in control cell lines without TGF-β_1 addition. Procollagen and fibronectin mRNA levels 24 hours after TGF-β_1 addition were elevated 3.6 fold and 19.6 fold, respectively. Interestingly, GAPDH mRNA levels were also slightly increased by TGF-β_1 (1.6 fold).

Discussion

In this report we have analyzed long term effects of TGF-β_1 on its own induction and on the induction of collagen and fibronectin mRNAs. Results indicate that 24 hour exposure of fibroblasts to TGF-β_1 under serum-free condition is sufficient to maintain elevated steady-state levels of TGF-β_1, collagen and fibronectin mRNAs for at least 96 hours (longer time points were not studied). Our data corroborates the findings by Varga et al (16) who have found elevated synthesis of collagens type I, III and fibronectin 72 hours after removal of TGF β. In contrast to our findings, these authors have found higher levels of matrix gene expression at 72 hours after TGF β removal than 24 hours after TGF β stimulation. These

differences may be due to the presence of fetal calf serum (5%) and ascorbate in their experimental system, as compared to serum-free defined medium utilized in our experiments.

It has been shown that secretion of active TGF-β_1 is present in the supernatants of human fibroblasts maintained under serum free conditions (3). Therefore, an attractive hypothesis explaining the presence of persistent elevated levels of TGF-β_1 mRNAs, in the absence of exogenous TGF-β_1, is the induction of a TGF-β autocrine loop. Such autostimulation of TGF-β, if present *in vivo*, suggests the possibility of long term effects of this growth factor on its target cells. In addition, collagen type I and fibronectin mRNA levels were persistently elevated which also may be due to the effects of autocrine TGF-β_1.

The regulation of expression of selected genes by TGF-β is not well understood, although both transcriptional and postranscriptional mechanisms have been identified (5,6,17). Effect of TGF-β on transcription and mRNA turnover in our experimental conditions is currently under investigation.

Compelling evidence has accumulated over the years to implicate cytokines and growth factors in the process of tissue repair and fibrosis. For example, PDGF, IL-1, TNF and TGF-β induce fibroblast proliferation (18,19,20,21), while IL-1, TNF and TGF-β induce matrix synthesis (19,22). However, TGF-β is the only known factor to induce all main components of extracellular matrix such as collagens type I and III, fibronectin and glycosoaminoglycans (4). Interestingly, the effects of TGF-β on the steady-state mRNA levels of extracellular matrix genes and c-myc gene mimic the *in vitro* spontaneous characteristics of lesional fibroblasts from the involved skin of scleroderma patients (23,24). It is possible that such a phenotype may be the consequence of the *in vivo* exposure of fibroblasts to TGF-β involving mechanisms similar to the persistant effects of TGF-β observed *in vitro*. To determine whether TGF-β is able to persistently activate fibroblasts *in vivo* by induction of a TGF-β autocrine loop, followed by increased synthesis of extracellular matrix would be important to understanding the role of this growth factor in the process of fibrosis.

These experiments are feasible with in situ hybridization and monoclonal antibody techniques.

Acknowledgments

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