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Effect of transforming growth factor-β on collagen type VI expression in human dermal fibroblasts

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Steady-state mRNA levels and protein synthesis of collagen type VI were determined after stimulation of human dermal fibroblasts with transforming growth factor-β (TGFβ). While there was a 227% increase in the α3(VI) subunit mRNA at maximal TGF-β concentration, α1(VI) and α2(VI) subunit mRNA levels remained unchanged. Concomitantly collagen type VI immuno-reactive material increased up to 172% of controls in cell culture medium and cell layer extracts. Regulation of α3(VI) gene expression is therefore critical for the control of collagen type VI synthesis and determines the deposition of collagen type VI heterotrimeric molecules.

1. INTRODUCTION

Collagen type VI is a structural component within the interstitial connective tissue of almost every organ (for review see [1]). Its molecular structure has been deduced from electron microscopy studies as well as from cDNA and protein sequencing [2]. Collagen type VI is composed of three individual α chains, α1(VI), α2(VI) (both of 140 kDa) and α3(VI) (340 kDa), with each being the product of a distinct single-copy gene. It is distinguished from other collagen types by a particularly short segment involved in triple helix formation and large globular domains at both ends [2]. The supramolecular assembly of collagen type VI represents a major part of the microfibrillar network within extracellular matrices [3,4]. Such microfibrils are often located around larger anatomical structures such as nerves, blood vessels and collagen fibers [5]. Increased amounts of collagen type VI have been observed in certain pathological conditions with disturbed connective tissue composition, such as multiple fibromatosis with articular dysplasia [6], neurofibromatosis [7] and keloids [8]. At present, however, little is known about the regulation of collagen type VI biosynthesis.

Collagen type VI is abundantly expressed in cultured human fibroblasts [9,10] and controlled by regulatory mechanisms independent from those regulating the interstitial collagen types I and III [11]. We have shown previously that interferon-γ selectively reduces the mRNA expression of only one subunit of collagen type VI, the α3(VI) chain. It was also demonstrated that this is sufficient to diminish the secretion and deposition of newly synthesized heterotrimeric molecules [12].

We now report on the effect of a potent collagen synthesis-inducing cytokine, transforming growth factor-β (TGF-β) [13], on specific mRNA and protein expression of all three collagen type VI chains. Our results indicate that the gene encoding the α3(VI) chain is the only target for this cytokine. This suggests that mechanisms regulating the synthesis of α3(VI) chain are crucial for the production of the entire collagen type VI molecule.

2. MATERIALS AND METHODS

2.1. *Cell cultures and TGF-β treatment*

Human skin fibroblasts were obtained by explantation of skin biopsies as described elsewhere [14], and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 50 μg/ml ascorbate, 300 μg/ml glutamine, 50 μg/ml streptomycin and 400 U/ml penicillin. Confluent fibroblast cultures between passages 4 and 9 were used for the following experiments.

Cells were washed with phosphate-buffered saline and then incubated for 24 or 48 h in fresh medium containing TGF-β (Paesel, Frankfurt, Germany) at concentrations ranging from 0 to 5 ng/ml. Medium was renewed after 24 h. For all experiments a separate set of culture dishes treated in the same manner was used to determine cell numbers (5.6–7.5 × 10^6 cells/dish).

2.2. *Determination of mRNA levels*

Total RNA was isolated from TGF-β-treated and untreated fibroblasts following established procedures [15]. Briefly, cells were collected in 4 M guanidine isothiocyanate followed by ultra-centrifuga-

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tion over a 5.7 M CsCl cushion. The resulting pellet was dissolved in water, extracted with phenol/chloroform, ethanol precipitated and re-dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 7. The amount of total RNA was determined photometrically at 260 nm. Northern blot and dot blot analysis using specific cDNA probes for the collagen chains, α1(I), α1(VI), α2(VI) and α3(VI), was carried out as described previously [12]. For the determination of β-tubulin mRNA a 264 bp cDNA fragment of the D.1 clone [16] was used.

2.3. *Determination of protein synthesis*

Control and TGF-β-treated fibroblasts were pulse labeled using 500 μCi/dish of [^{3}H]proline (Amersham, 3300 Braunschweig, Germany) in serum-free, otherwise identical medium during the final 24 h of incubation. Culture media were collected on ice and frozen immediately at −20°C after addition of protease inhibitors (0.5 mM phenylmethane sulfonyl fluoride, 5 mM *N*-ethylmaleimide, 1 mM EDTA). Cell monolayers were subjected to extraction and analyzed by immuno-dot binding assay as previously described [12] using antibodies raised against collagen VI [4]. Incorporation of [^{3}H]proline in newly synthesized material was determined by measuring the radioactivity of ethanol-precipitated proteins from the culture medium and the cell layer extracts. Labeled culture medium was treated with pepsin (0.1% in 0.5 M acetic acid, 6 h at 4°C) and analyzed by SDS-PAGE on 10–20% acrylamide gradient gels after reduction with β-mercaptoethanol. The gels were processed for fluorography as described previously [12] and exposed to X-ray films.

3. RESULTS

3.1. *Collagen type VI mRNA steady-state levels*

Treatment for 48 h of confluent human dermal fibroblasts with TGF-β resulted in a selective increase of the mRNA coding for the α3(VI) chain of collagen type VI as observed by Northern blot analysis (Fig. 1). In contrast, levels of the mRNAs coding for the α1(VI) and α2(VI) chains were not affected (Fig. 1). The effect of TGF-β on α3(VI) mRNA was dose-dependent, as shown by dot blot analysis of specific mRNA levels after exposure of the cells to different concentrations of TGF-β (Fig. 2). The maximal increase of α3(VI) mRNA (227%) by TGF-β (5 ng/ml, 48 h) was comparable to that of another collagen chain, α1(I) mRNA (219%) under identical conditions (Table I). An incubation period of 24 h with TGF-β resulted in a similar, but less pronounced selective increase of the α3(VI) and α1(I) mRNA levels (Table I). These effects were consistently observed in the presence or absence of fetal calf serum (not shown). Expression of β tubulin, an intracellular

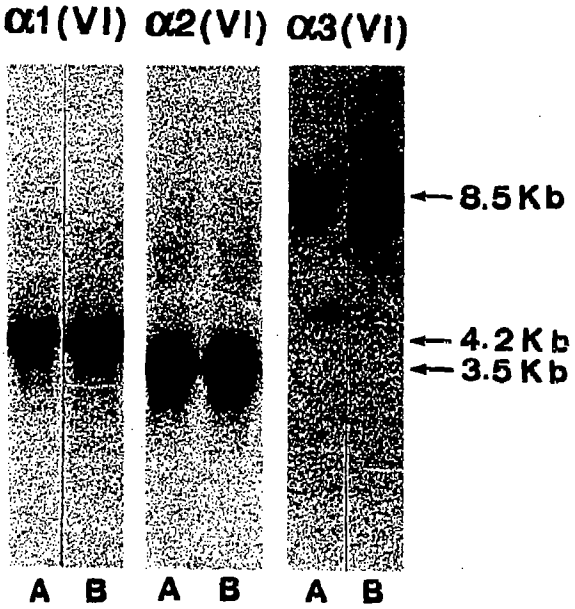

Fig. 1. Northern blot analysis of collagen type VI-specific mRNAs in TGF-β-treated fibroblasts. Confluent human dermal fibroblasts were incubated for 48 h without (A) or with (B) TGF-β (5 ng/ml). Total RNA was separated by gel electrophoresis as described in section 2 and transferred onto nitrocellulose. Filters were hybridized with ^{32}P-labeled cDNA probes specific for α1(VI), α2(VI), and α3(VI) chains, yielding signals of the expected sizes [9].

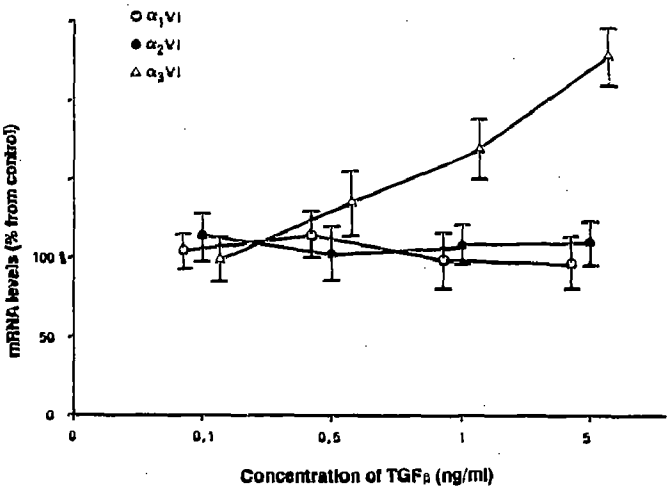

Fig. 2. TGF-β dose-dependent mRNA-levels of collagen type VI subunits. Confluent human dermal fibroblasts were incubated with various concentrations of TGF-β for 48 h. Total RNA was isolated and specific mRNA levels for α1(VI) (○), α2(VI) (●), and α3(VI) (△) were determined by dot blot analysis as described in section 2. After densitometric scanning of radiographs, results were calculated as the % of untreated controls. Results are expressed as means ± S.D. of three independent experiments.

Table I

Collagen mRNA levels in fibroblasts after TGF-β treatment

mRNA analyzed	Duration of TGF-β treatment	
	24 h	48 h
Collagen type I α1(I)	169 ± 15	219 ± 21
Collagen type VI α1(VI)	104 ± 12	96 ± 17
Collagen type VI α2(VI)	98 ± 15	109 ± 14
Collagen type VI α3(VI)	162 ± 19	227 ± 19
β-Tubulin	110 ± 16	114 ± 18

Cells were treated for 24 and 48 h with TGF-β (5 ng/ml). Specific mRNA levels were determined by dot blot analysis and are expressed as the % of control values obtained with untreated cells (=100%). Results are expressed as mean ± S.D. of three independent experiments.

Table II

Protein synthesis in TGF-β-treated human skin fibroblast cultures

TGF-β (ng/ml)	[^{3}H]Proline incorporation			Collagen type VI (%) in	
	Total (cpm/10^3 cells)	Medium (%)	Cell layer (%)	Medium	Cell layer
0	3,648 ± 196	62	38	100	100
0.5	5,200 ± 801	56	44	147	132
5	5,177 ± 897	46	54	177	168

Human skin fibroblasts were incubated for 48 h with the indicated concentrations of TGF-β. Incorporation of [^{3}H]proline was determined by measuring the radioactivity of ethanol-precipitable material in culture media and cell layers. Collagen type VI production was evaluated by immuno-dot binding assay [11] and the results were expressed in % compared to untreated cells set at 100%. All values are the mean of three separate experiments.

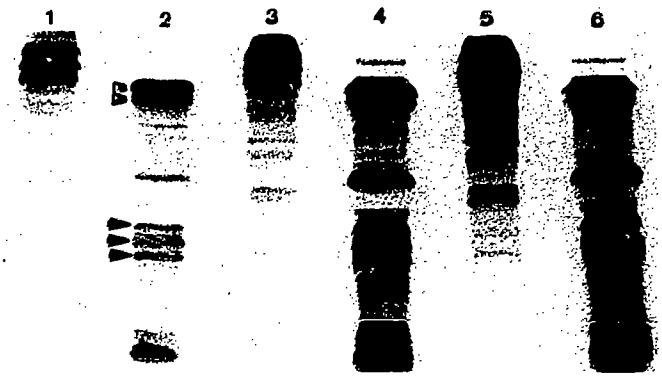

Fig. 3. Electrophoretic analysis of labeled culture medium from control and TGF-β-treated fibroblasts. Samples were used without (lanes 1, 3 and 5) or after (lanes 2, 4 and 6) pepsin treatment. (Lanes 1 and 2) control fibroblasts; (lanes 3 and 4) 0.5 ng/ml TGF-β-treated fibroblasts; (lanes 5 and 6) 5 ng/ml TGF-β-treated fibroblasts. Open arrowheads denote the position of collagen type I chains. Closed arrowheads denote the position of pepsin-resistant collagen type VI chains (from top to bottom, α1(VI), α2(VI) and α3(VI) chains).

constitutive protein, was only slightly increased to 121% at the maximal TGF-β concentration, indicating that the TGF-β-induced changes of collagen synthesis are specific (Table I).

3.2. *Collagen type VI and protein synthesis*

After incubation of fibroblasts with TGF-β (5 ng/ml), total protein synthesis estimated by [^{3}H]proline incorporation increased up to 142% of untreated cells (Table II). In addition there was a shift in the distribution of radiolabeled material between culture medium and cell layers (Table II) indicating that TGF-β-treated cells incorporate more of the newly synthesized material into the cell layer than untreated cells.

Immunological analysis also showed a maximal increase of 172% for collagen VI after TGF-β treatment (Table II). This increase was observed for both the material secreted into the culture medium and that deposited into the cell layer. An increase in type VI collagen production was also demonstrated by electrophoresis after metabolic labeling of cell culture medium. Pepsin digestion followed by reduction showed the characteristic triplet bands of collagen VI in the control medium and a distinct increase in all three bands after TGF-β treatment (Fig. 3). This strongly indicates that TGF-β causes increased production of collagen VI containing all three chains in stoichiometric proportions [9].

4. DISCUSSION

Many studies have shown that TGF-β up-regulates the expression of the fibril-forming collagen types, I and III, of fibronectin, proteoglycans and several collagen-binding integrin receptors [13,17–20]. In addition it lowers the production of matrix metalloproteases and increases that of their inhibitors [21]. These effects of the cytokine point to a central role in the regulation of extracellular matrix deposition by increasing synthesis and interfering with the catabolic mechanisms [13]. Our data add collagen type VI to the list of matrix components up-regulated by TGF-β even though only the α3(VI) chain seems to be involved at the mRNA level. For fibroblasts grown at different cell densities or in a collagen gel it was previously shown that the synthesis of collagen VI is independently regulated from that of collagen types I and III [11]. TGF-β apparently imposes a new regulatory mechanism and forces the synthesis of all the collagens examined so far in fibroblasts in the same direction. Presumably this occurs by the induction of *trans*-acting factors which are still not well defined [22]. A related phenomenon was observed in fibroblasts treated with interferon-γ [12] which reduces similarly expression of collagen types I, III and VI, in the latter case by a selective down-regulation of α3(VI) mRNA. This suggests that the human α3(VI) chain gene, which is located on chromosome 2q37 [23], plays a crucial regulatory function in collagen type VI synthesis. The α1(VI) and α2(VI) chain genes are syntenic on chromosome 21q23 and apparently co-regulated in fibroblasts and some tumor cells [9,11]. Their expression is not affected by TGF-β, as shown here and by interferon-γ [12].

Steady-state mRNA levels of α1(VI), α2(VI) and α3(VI) show an approximate 1:1:1 ratio in cultured fibroblasts [9,11]. The down-regulation of α3(VI) mRNA by interferon-γ causes a reduced synthesis and deposition of collagen type VI [12], which is in agreement with structural predictions [24] that only trimeric molecules possessing all three chains are the genuine and most stable assembly products. Even though those chains produced in excess can be secreted, they are not properly stabilized against pepsin treatment and not deposited in the cell matrix [12]. The steady-state mRNA

levels in fibroblasts may, however, not necessarily reflect the levels of newly synthesized chains. The data shown here suggest a more efficient translation of α1(VI) and α2(VI) compared to α3(VI) chains which, when compensated by a TGF-β-mediated increase in α3(VI) mRNA, allows for the increased production of stable collagen type VI molecules. This rescues the surplus of α1(VI) and α2(VI) chains in a pepsin-stable form, as demonstrated in Fig. 3. There is also some evidence that similar processes may occur in situ. A strong induction of α3(VI) mRNA is seen during early phases of wound healing, which declines at later stages before the increased expression of α1(VI) and α2(VI) mRNA [25]. A spatial and temporal co-expression was also observed for TGF-β and collagen type VI in human keloids [8]. Our data therefore suggest a mechanism which may be of relevance for connective tissue deposition during repair processes and fibrosis.

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