Regulation of Fibronectin and Type I Collagen mRNA Levels by Transforming Growth Factor-β*

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Human platelet-derived transforming growth factor-β (TGF-β1) increases the accumulation of the extracellular matrix proteins, fibronectin and type I collagen, in mesenchymal and epithelial cells. To determine the basis for this effect, we have examined the levels of mRNAs corresponding to fibronectin and α2(I) procollagen in NRK-49F rat fibroblasts and L4E6 rat myoblasts treated with TGF-β1. TGF-β1 increased severalfold the levels of mRNAs for both proteins. The kinetics of this effect were similar for both mRNAs. The increase in fibronectin and α2(I) procollagen mRNAs was detectable 2 h after addition of TGF-β1 to the cells and their maximal levels remained constant for several days. Actinomycin D, but not cycloheximide, inhibited the increase in fibronectin and α2(I) procollagen mRNA levels induced by TGF-β1. The results indicate that TGF-β1 controls the composition and abundance of extracellular matrices at least in part by inducing a coordinate increase in the levels of fibronectin and type I collagen mRNAs.

Type β transforming growth factors (TGF-β) are hormonally active polypeptides consisting of two 12-kDa chains linked by disulfide bonds (reviewed in Refs. 1 and 2). They were found in a wide range of normal and transformed cells (3, 4) being particularly abundant in platelets (5, 6) and bone (7). Two homodimeric forms of TGF-β, termed TGF-β1 and TGF-β2, have been identified to date. These forms are structurally and biologically related but display only about 70% amino acid sequence identity in the 43 amino-terminal residues and show additional differences in the carboxyl-terminal domains (6). Porcine platelets contain TGF-β1 and TGF-β2 homodimers as well as the heterodimer, TGF-β1.2 (6). To date, only TGF-β1 has been identified in human platelets (5). Initial characterization of TGF-β was based on its effects on cell proliferation. TGF-β is from various sources allow anchor-

dependent growth of untransformed fibroblasts (2) and promote monolayer growth under certain conditions (8). The effect on cell proliferation. TGF-β is prototypic of a larger family of polypeptide factors that controls tissue development in organisms from Drosophila to humans. In addition to TGF-β1 and TGF-β2, this family includes inhibins and activins that control pituitary cell functions (17), Müllerian inhibiting substance that controls the development of the Müllerian duct in mammalian embryos (18), and the decapentaplegic transcript critically involved in various stages of Drosophila development (19). Because of their high abundance in platelets, bone, and developing tissues (20), TGF-βs are likely to function in a broad range of activities involved in tissue development and repair. We have examined proteins of the extracellular matrix as biochemical targets of TGF-β action because of the known ability of the extracellular matrix to influence the expression of individual phenotypes. TGF-β1 and TGF-β2 increase the synthesis of fibronectin (12, 21) and collagen (12, 21, 22) by many cell types in culture or in vivo (22, 23). In addition, TGF-β1 appears to regulate the production of proteins that can modify the extracellular matrix by proteolytic action, such as plasminogen activator and an inhibitor of plasminogen activator (24), procollagenase (25), and several other secreted proteins of as yet unknown function (26). Thus, TGF-βs might control the expression of specific phenotypes in vivo by regulating the composition of extracellular matrices.

Previous reports concerning the modulation of extracellular matrix components of TGF-β have dealt with the measurement of the rates of accumulation of fibronectin and type I collagen (21, 22). This effect of TGF-β could be mediated by changes in the rates of synthesis and/or stabilization of the various proteins. In this report, we show evidence that TGF-β1 controls the levels of fibronectin and type I collagen, at least in part, by increasing the levels of the respective messenger RNAs. The increase in fibronectin and type I collagen mRNA levels is an early response of cells to TGF-β and lasts as long as the factor is present in the medium.

MATERIALS AND METHODS

The sources and culture conditions of NRK-49F rat fibroblasts and L4E6 rat myoblasts have been described previously (3, 21). TGF-β1 was purified to homogeneity from outdated human platelets as described previously (15). Total cellular RNA was extracted essentially as described by Chirgwin et al. (27) from subconfluent cultures of NRK-49F cells that had been treated with various reagents. To assure that equal amounts of poly(A)* RNA were subjected to Northern blot analysis, portions of the total RNA samples were subjected to hybridization to [3H]polyuridylic acid. Briefly, 10–15 μl of the sample were...
incubated with [3H]polyuridylic acid (Du Pont-New England Nuclear) in 2 x SSC (SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) for 45 min at 30 °C. Nonhybridized radioactivity was removed by digestion of the incubation mixtures with 40 μg/ml RNase A for 30 min at room temperature. Samples were then precipitated by addition of 10% trichloroacetic acid with bovine serum albumin as a carrier. The precipitates were collected on filters and the radioactivity was measured. RNA samples were adjusted such that amounts of RNA giving equivalent hybridization to the [3H]poly(U) were loaded onto the gels. The discrepancy between RNA values quantitated by A260 absorbance and [3H]poly(U) hybridization was usually less than 10%. Total cytoplasmic RNA from NRK cells was isolated as previously described (12).

RNA samples were fractionated by the size on 1% agarose gels containing 2.2 M formaldehyde and transferred to nitrocellulose filters as described (12). The filters were hybridized with nick-translated cDNA probes and processed for autoradiography as described previously (12). The rat fibronectin cDNA (rLF-1) was generously provided by Richard Hynes (Massachusetts Institute of Technology) and the chick α2(I) procollagen cDNA (pCG45) was kindly provided by Helga Boedtker (Harvard University).

RESULTS

We examined the effect of TGF-β1 on the levels of mRNAs corresponding to fibronectin and type I collagen in NRK-49F fibroblasts, a cell line responsive to TGF-β. Fig. 1 shows Northern blot analyses of RNA from NRK-49F cells treated with TGF-β1 for 12 or 24 h. A higher level of hybridization of fibronectin as well as α2(I) procollagen cDNA probes was observed in the samples from TGF-β1-treated cells. The molecular size of the hybridizing species, 8 and 5.2 kilobases, were those expected for mRNAs corresponding to rat fibronectin and α2(I) procollagen (28,29), respectively. Thus, the increase in fibronectin and type I collagen proteins is correlated with an increase in the respective mRNAs in response to TGF-β.

Fig. 1. Analysis of mRNA levels for fibronectin and α2(I) collagen following treatment with TGF-β. NRK cells were grown to near confluency and then placed into serum-free medium with or without 500 pm TGF-β. After either 12 or 24 h, cultures were harvested and total RNA was extracted and isolated according to Chirgwin et al. (27). Equal amounts of poly(A)+ RNA were analyzed, based on quantitation by hydridization to [3H]poly(U) were loaded onto the gels. The kinetic measured. RNA samples were adjusted such that amounts of RNA giving equivalent hybridization to the [3H]poly(U) were loaded onto the gels. The discrepancy between RNA values quantitated by A260 absorbance and [3H]poly(U) hybridization was usually less than 10%. Total cytoplasmic RNA from NRK-49F cells was isolated as previously described (12).

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The kinetics of the increase of fibronectin and α2(I) procollagen mRNA levels are shown in Fig. 2A. An increase in the levels of these mRNAs was detectable by 2 h, reached a maximum at approximately 10 h, and remained constant for at least 24 h following a single addition of TGF-β1 to the cultures. These kinetics are similar to those of increased production of the proteins (21). The effect of TGF-β1 was concentration-dependent, being half-maximal at 25 pm TGF-β1 (Fig. 2B). Thus, the elevation of fibronectin and type I collagen mRNAs in NRK-49F fibroblasts is an early, persistent, and potent effect of TGF-β1.

The mechanism of the increase in fibronectin and α2(I) procollagen mRNA in response to TGF-β is not known. However, treatment of cultures with the inhibitor of transcription, actinomycin D, blocked the increase in fibronectin (Fig. 3) and α2(I) procollagen (not shown) mRNAs induced by TGF-β1. This suggests, but does not unequivocally demonstrate, the involvement of transcriptional events in the response. To assess the possible role of protein synthesis in this action of TGF-β1, cultures were treated with TGF-β1 and the protein synthesis inhibitor, cycloheximide, concomitantly. In the presence of cycloheximide, TGF-β1 was still able to increase the mRNA levels (Fig. 3). This result suggests that de novo protein synthesis is not required for the induction of fibronectin and collagen mRNAs by TGF-β1.

Fig. 2. Parameters of the TGF-β effect on fibronectin and collagen mRNA levels. A, cultures of NRK cells were placed into serum-free medium supplemented with 500 pm TGF-β. After the indicated lengths of time, cultures were harvested and total cellular RNA was isolated and analyzed by Northern blotting with hybridization to either the fibronectin probe (●) or the α2(I) collagen probe (O). The blots were subjected to autoradiography, and the relative intensity of the autoradiographic signal was determined by densitometry. The results are expressed as the ratio of mRNA levels in TGF-β1-treated versus untreated cells. B, cultures of NRK cells were placed into serum-free medium supplemented with varying concentrations of TGF-β. After 12 h, the RNA was extracted and subjected to Northern blot analysis for fibronectin (●) and collagen (O) mRNAs. The resulting autoradiographs were analyzed by densitometry. The results are expressed as indicated above.
Another cell type examined in these studies was L6E9 rat skeletal muscle myoblasts. Like other lines and primary cultures of myoblasts, L6E9 cells are inhibited by TGF-β1 in their ability to undergo terminal differentiation to form myotubes (12). TGF-β prevents expression of the muscle-specific mRNAs and proteins, α-actin, myosin heavy and light chains, α- and β-tropomyosin, and troponin subunits in these cells (12) (see also Fig. 4A). L6E9 cells and the related clones retain their undifferentiated phenotype of myoblasts as long as TGF-β is present in the medium (12) (Fig. 4A). In addition, it has been shown that manipulation of the extracellular matrix of these cells by addition of exogenous fibronectin inhibits the expression of the muscle phenotype (30) and that treatment of these cells with TGF-β increases the production and accumulation of fibronectin and collagen (12, 21). Thus, rat skeletal muscle myoblasts provide an advantageous system to study the role of the extracellular matrix in controlling expression of developmentally regulated muscle-specific genes and commitment to terminal differentiation. We examined the levels of fibronectin and type I collagen mRNAs, comparing them with those of muscle-specific mRNAs and of β- and γ-actin mRNAs, during inhibition of myogenogenesis by TGF-β. TGF-β1 elevated fibronectin and α2(I) procollagen mRNAs strongly (Fig. 4B). The effect lasted as long as TGF-β1 was present in the medium and disappeared gradually after the transfer (rescue) of the differentiation-blocked cells to normal differentiation medium lacking TGF-β1. The progressive decrease in the levels of these mRNAs was observed during differentiation of L6E9 cells (Fig. 4). This is in agreement with previous observations in their related myoblast cell lines (31). Interestingly, while fibronectin and collagen mRNAs were greatly induced in TGF-β1-treated myoblasts, the levels of β- and γ-actin mRNAs declined to some extent in the presence of TGF-β1 (Fig. 4). This finding is in contrast with the TGF-β-induced increase in β- and γ-actin mRNA levels in AKR-2B mouse fibroblasts (32).

**DISCUSSION**

The altered expression of fibronectin and type I collagen in transformed cells offers a precedent for the involvement of transcriptional as well as post-transcriptional changes in the regulation of fibronectin and collagen expression. For example, decreased transcription of fibronectin and type I collagen genes has been observed in cells transformed by Rous sarcoma virus or the oncogene v-mos (28, 33-35). The levels of these proteins can also change due to alterations in their stability (36). Thus, different ways might exist by which TGF-βs exert their previously observed effects on fibronectin and type I collagen expression.

The results described here imply that TGF-β1 increases the synthesis of fibronectin and type I collagen at least in part by increasing the levels of their corresponding mRNAs. Events sensitive to actinomycin D appear to be required for this response. These observations together with results from previous biosynthetic labeling experiments (21, 22) indicate that increased synthesis of fibronectin and type I collagen is a major component of the action of TGF-βs on extracellular matrices. These results do not exclude, however, the possibility that additional control by TGF-βs could be exerted at the level of post-transcription, translation, or the stabilization of the proteins.

The parallel kinetics of the effects of TGF-β1 on fibronectin and type I collagen mRNA levels point to the possibility that TGF-β controls the activity of cellular elements that regulate in a concerted fashion the expression of fibronectin and type I collagen. TGF-β could accomplish this effect by a variety of mechanisms, but the results obtained with cycloheximide argue against the possibility that TGF-β acts by increasing the synthesis of proteins involved in regulating fibronectin and type I collagen mRNA levels.

The possible consequences of altering the composition and abundance of extracellular matrices in fibroblasts, myoblasts, and other cell types of TGF-βs in *vivo* remain to be established. However, it has been found that induction of anchor-angle-independent growth of cultured NRK-49F cells by TGF-β is preceded by accumulation of cell-derived fibronectin in...
the soft agar cultures and can be inhibited by synthetic peptides that prevent the assembly of fibronectin-based matrices (21). Likewise, a causal link appears to exist between changes in the extracellular matrix of myoblasts and preadipocytes induced by TGF-β1, and the ability of this factor to prevent myogenic and adipogenic differentiation (11, 12). These observations imply that control of the composition and abundance of extracellular matrices might be a central component of the action of TGF-β in physiological processes of tissue development and remodeling in which these factors intervene.

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REFERENCES

1. Massagué, J. (1985) Trends Biochem. Sci. 10, 237–240
2. Sporn, M. B., Roberts, A. B., Wakefield, L. M., and Assoian, R. K. (1986) Science 233, 532–534
3. Assoian, R. K., Roberts, A. B., Wakefield, L. M., Anzano, M. A., and Sporn, M. B. (1985) Cancer Cells Growth Factors and Transformation, pp. 59–64, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
4. Massagué, J. (1984) J. Biol. Chem. 259, 9756–9761
5. Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M., and Sporn, M. B. (1985) J. Biol. Chem. 260, 7155–7160
6. Cheifetz, S., Weaver, J. A., Tsang, M., L. S., Anderson, J. K., Molé, J. E., Lucas, R., and Massagué, J. (1987) Cell 48, 409-415
7. Seyedin, S. M., Thompson, A. Y., Bentz, H., Rosen, D. M., McPherson, J. M., Conti, A., Siegel, N. R., Galluppi, G. R., and Piez, K. A. (1986) J. Biol. Chem. 261, 5693–5695
8. Shipley, C. D., Tucker, R. F., and Moses, H. L. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4147–4151
9. Tucker, R. F., Shipley, G. D., Moses, H. L., and Holley, R. W. (1984) Science 226, 705–707
10. Roberts, A. B., Anzano, M. A., Wakefield, L. M., Roche, N. S., Stern, D. F., and Sporn, M. B. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 119–123
11. Ignotz, R. A., and Massagué, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8530–8534
12. Massagué, J., Cheifetz, S., Endo, T., and Nadal-Ginard, B. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8296–8300
13. Rosen, D. M., Stempien, S. A., Thompson, A. Y., Brennan, J. E., Ellingsworth, L. R., and Seyedin, S. M. (1986) Exp. Cell Res. 165, 127–130

1 R. A. Ignotz and J. Massagué, unpublished observations.