Transforming Growth Factor-β Induces Plasminogen Activator Inhibitor Type-1 in Cultured Human Orbital Fibroblasts

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Purpose. The level of constitutive plasminogen activator inhibitor type-1 (PAI-1) expression in cultured human orbital fibroblasts is considerably lower than that found in dermal fibroblasts. This divergence in PAI-1 expression implies differences in the pericellular proteolytic environment and, therefore, in the turnover of extracellular matrix. In this article, the authors examine the effect of transforming growth factor-β (TGF-β) on PAI-1 expression in orbital fibroblasts.

Methods. Human orbital and dermal fibroblasts were grown in culture. Confluent monolayers were treated with TGF-β. PAI-1 in the extracellular matrix was quantitated by radiolabeling the cultures and electrophoresing the cellular material on SDS-PAGE. Medium content was determined by immunoprecipitation of [35S] PAI-1 with a rabbit, anti-human, polyclonal antibody. PAI-1 mRNA was determined by Northern hybridization.

Results. TGF-β increased PAI-1 levels in orbital fibroblasts in a dose-dependent manner, up to 35-fold. The induction was maximal after 16 hours of treatment. The increases in extracellular matrix PAI-1 paralleled those observed in the medium. The steady state levels of the mRNA encoding the protein were upregulated by TGF-β up to 60-fold 8 hours after the addition of TGF-β. The fractional increase in PAI-1 expression in orbital fibroblasts was consistently greater than that observed in dermal strains.

Conclusions. Exposure to TGF-β consistently induces PAI-1 expression in orbital fibroblasts, cells that do not express the polypeptide constitutively at high levels. The effects are mediated at the pretranslational level and involve the upregulation of PAI-1 mRNA. These results suggest that TGF-β may exert a profound regulatory influence on the pericellular proteolytic environment in orbital connective tissue. Invest Ophthalmol Vis Sci. 1995;36:1411–1419.

Human orbital fibroblasts maintained in primary culture exhibit phenotypic attributes setting them apart from those derived from dermis. In particular, responses to a variety of molecular factors are different in orbital cultures.1–6 We reported recently7 that recombinant interferon γ can increase the expression of plasminogen activator inhibitor type-1 (PAI-1), an abundant serine protease inhibitor, in orbital fibroblasts. These cells do not synthesize high levels of the polypeptide constitutively.7,8 In contrast, depending upon the anatomic region of origin, the lymphokine either decreases or only slightly increases PAI-1 synthesis in dermal cultures.7–10 PAI-1 is an important determinant of the pericellular proteolytic environment,11,12 and, therefore, its differential expression in orbital fibroblast cultures might reflect a characteristic of extracellular matrix (ECM) turnover in the orbit that sets this connective tissue apart from that found elsewhere.

Transforming growth factor-β (TGF-β) influences a number of cellular processes in a wide variety of target tissues.13 This cytokine possesses diverse functions as an autocrine–paracrine factor. Cell differentiation and locomotion, angiogenesis, adhesion, and proliferation are influenced by TGF-β and other structurally related factors.13–16 The actions ascribed to TGF-β are elicited through high-affinity binding to a number of recently identified and characterized re-
ceptor proteins. Recent evidence suggests the requirement for heterodimeric structure for full biological activity of these receptors. Transforming growth factor-β alters the expression of key components of the ECM. It has been shown to be capable of increasing PAI-1 expression in established cell lines in vitro, including WI-38 lung fibroblasts. There is no information concerning the influence of TGF-β on PAI-1 expression in primary human fibroblasts. The insight that human fibroblasts can express the cytokine and that physiological states such as hypoxia induce its synthesis suggests a potential role for TGF-β as an autocrine factor for fibroblasts expressing it. Thus, TGF-β might represent an endogenous regulator of PAI-1 expression and, hence, ECM turnover in the fibroblast.

In this article, we report that TGF-β can increase the expression of PAI-1 in cultured orbital fibroblasts substantially. This effect is mediated by increases of the steady state levels of mRNA encoding this polypeptide. Our current findings identify another potentially important regulatory factor of ECM stability in orbital connective tissue that might be relevant to the pathogenesis of diseases involving the accumulation of interstitial material. An example of such a process is the ophthalmopathy associated with Graves’ disease.

MATERIALS AND METHODS

Materials

Recombinant human TGF-β1 and culture reagents were supplied by Grand Island Biological Company (Grand Island, NY). (35S)Methionine (specific activity > 1100 Ci/mmol) was purchased from New England Nuclear (Boston, MA). A rabbit polyclonal antibody directed against human PAI-1 was obtained from American Diagnostica (Greenwich, CT). All other reagents used were of the highest purity commercially available.

Cell Culture

Fibroblast cultures were initiated from tissue explants obtained during orbital surgery for severe Graves’ ophthalmopathy or some other disease process not involving the orbit. Dermal fibroblast cultures were obtained from punch biopsies of normal-appearing skin of people without known thyroid disease or from patients with Graves’ disease. Four orbital connective tissue strains from patients with Graves’ ophthalmopathy, three orbital connective tissue strains from persons without orbital disease, three strains of perimysial fibroblasts, three abdominal dermal strains, and one prethbial dermal strain were used in these studies. Some of the material was kindly provided by Drs. R. Dallow (Harvard Medical School, Boston, MA), D. Meyer (Albany Medical College, Albany, NY), and R. Bahn (Mayo Clinic, Rochester, MN). Methods for securing human tissue were humane, tenets of the Declaration of Helsinki were followed, and institutional review board approval was obtained for these activities. After the initiation of a strain, aliquots of material were stored at −70°C until needed. Monolayers were allowed to proliferate to confluence, as reported, in 35-mm diameter plastic culture dishes covered with Eagle’s medium supplemented with 10% fetal calf serum, antibiotics, and glutamine. Cultures were maintained in a humidified, 5% CO2 incubator at 37°C, and the medium was changed every 3 to 4 days. For the experiments shown, cultures were used between the 6th and 10th passages from initiation. We have demonstrated that PAI-1 expression and inducibility are constant during this period. Confluent cultures were shifted to medium containing the cytokine for the treatment times indicated in the legends to the figures. Metabolic labeling was conducted in methionine-free, serum-free RPMI 1640 medium supplemented with 10% serum, to which 50 μCi/ml of [35S]-methionine was added. Labeling followed extensive washing of the monolayers with phosphate-buffered saline (PBS) and usually lasted 3 hours. Cultures treated with TGF-β were labeled in the presence of the compound.

Quantitation of PAI-1

These procedures have been published in detail. Briefly, medium used for the metabolic labeling of PAI-1 was removed quantitatively and clarified by centrifugation at 13,000g at 4°C. The cell layers were washed extensively with PBS and treated with 0.1% deoxycholate in PBS, the cellular material was washed away, and the matrix was solubilized with a solution containing 1% sodium dodecyl sulfate, 1% β-mercaptoethanol, 10% glycerol, and 50 mM Tris-HCl, pH 6.8. The matrix material was mobilized with a rubber policeman and frozen until assayed. Equivalent fractions of each sample, based on matrix protein content, were subjected to polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli.

Aliquots of medium were subjected to immunoprecipitation with a polyclonal antibody against PAI-1 by adding 1.5 μl of the rabbit anti-serum to 300 μl of medium. After a 2-hour incubation at 4°C, 50 μl of prewashed protein A (Calbiochem, La Jolla, CA) was added to each sample for another 2 hours at 4°C. Samples were then centrifuged at 14,000g. The resultant pellet was washed three times in a buffer containing 0.5% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, and 10 mM Tris-HCl, pH 7.5, and boiled for 5 minutes. Each specimen was handled quantitatively, and the entire sample was loaded on the gel.

All samples were subjected to PAGE in 10% polyacrylamide gels impregnated with EN3Hance (New
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FIGURE 1. The effect of increasing concentrations of TGF-β on the synthesis of proteins associated with the extracellular matrix. Confluent cultures of orbital fibroblasts were treated with graded concentrations of the cytokine for 16 hours and then were subjected to metabolic labeling with [35S]methionine (50 μCi/ml) for 3 hours. Matrices were processed as described in Materials and Methods, and the material was subjected to PAGE with 10% polyacrylamide and autofluorography. The concentrations used were as follows (in ng/ml): lane 1, control; lane 2, 0.025; lane 3, 0.05; lane 4, 0.125; lane 5, 0.25; lane 6, 0.5; lane 7, 0.75; lane 8, 1; lane 9, 5; lane 10, 10; lane 11, 15. The cultures were derived from a patient with severe Graves' ophthalmopathy.

Quantitation of mRNA Encoding PAI-1
The abundance of PAI-1 mRNA was measured by Northern blot analysis. Total cellular RNA was extracted from confluent fibroblast monolayers by the method of Chomczynski and Sacchi.25 The RNA (5 μg of each sample) was electrophoresed on agarose–formaldehyde gels, transferred to Zetaprobe membrane (BioRad, Hercules, CA), and allowed to hybridize with a human PAI-1 cDNA probe26 that was [32P]-dCTP-labeled by the random primer method. The filter was exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY) at —70°C. Fluorograms were developed, and band intensities were determined with a Bioimage gel scanner (Milligen, Bedford, MA).

RESULTS
Dose Response
As Figure 1 suggests, cultured orbital fibroblasts express low levels of PAI-1 protein, consistent with previous observations.14 The addition of graded concentrations (0 to 15 ng/ml) of TGF-β increased dramatically the expression of an approximately 50-kd protein associated with the extracellular matrix (Fig. 1). There was a greater than twofold increase with 0.1 ng/ml and an eightfold increase with 0.75 ng/ml. The presence of higher concentrations failed to produce further increases in the expression of this protein band.

Time Course of the Effects of TGF-β on PAI-1 Expression
The time dependence of effects of TGF-β on de novo protein synthesis of ECM proteins in orbital fibroblasts is shown in Fig. 2. Synthesis of a number of low-abundance proteins appears to be influenced by the cytokine. A major protein induction involves the region around 50 kd, where a faint signal is present at time 0 (untreated control), but which by 4 hours is induced 11.5-fold. The induction is maximal at 16 hours, where it is 21-fold above baseline values in control cultures. In contrast to orbital cultures, untreated dermal fibroblasts express the protein band strongly (Fig. 3). The addition of TGF-β to the culture medium increases the intensity of the band modestly at 8 hours of treatment when levels were threefold above control. A fivefold induction of the protein band was achieved at 16 and 24 hours after the addition of TGF-β to the dermal cultures. It is important to note that the fibroblasts used for these two time-course studies, represented by Figures 2 and 3, are derived from the same donor. This suggests strongly that differences in PAI-1 expression among individual subjects cannot explain the systematic differences in PAI-1 expression and inducibility observed in orbital and dermal culture strains.

Beside the major induction of the approximately 50-kd protein in orbital fibroblasts, TGF-β appears to have some influence on the expression of several low-abundance species associated with the extracellular matrix. As Figure 2 suggests, there may be some loss of a protein running above the 80-kd standard after 8 hours of exposure to the cytokine. There may also be an attenuation of a protein just above the PAI-1 protein band and a loss of the major 60-kd protein at 24 hours.

To identify the induced 50-kd protein positively as
PAI-1, aliquots of medium were subjected to immuno-precipitation with an antibody directed against the human polypeptide. As Figure 4 demonstrates, a single distinct band of radioactivity co-migrates with that shown in Figures 2 and 3 at approximately 50 kd and is induced dramatically by TGF-β so that by 8 hours, the intensity of the band is 31-fold higher than in the control sample. At 24 hours, the level of PAI-1 expression is increased 36-fold. The low abundance of the polypeptide in the

![Figure 2](image1)

**Figure 2.** Time dependence of the effect of TGF-β on the synthesis of proteins associated with the extracellular matrix in orbital fibroblast cultures from a patient with severe Graves' ophthalmopathy. Confluent cultures were treated for the intervals indicated along the abscissa with the cytokine (2 ng/ml). They were labeled metabolically with [35S]methionine as described in Materials and Methods. (bottom panel) Densitometric analysis of the 50-kd region of the gel.

medium of untreated orbital cultures is consistent with results published previously by us.7

**Pulse-Chase Studies**

Confluent orbital fibroblast cultures were incubated in the absence or presence of TGF-β (2 ng/ml) for 16 hours and then pulse-labeled with [35S]methionine for 3 hours This was followed by incubation in medium devoid of the radiolabeled precursor (chase), and cultures were harvested at the times indicated along the abscissa of Figure 5. The ECM was processed and subjected to PAGE, and the band corresponding

![Figure 3](image2)

**Figure 3.** Time dependence of the effect of TGF-β on the synthesis of matrix proteins in confluent dermal fibroblast cultures. The study was performed as described in the legend to Figure 2. Cultures derive from abdominal skin of the same donor as orbital fibroblasts used in the study depicted in Figure 2. (bottom panel) Densitometric analysis of the 50-kd region of the gel.
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HOURS 0 3 4 8 16 24

Mr: kDa

49.5 =>

FIGURE 4. Effect of TGF-β on radioactive material immunoprecipitated from culture medium of an orbital fibroblast strain with a polyclonal antibody directed against human PAI-1. Aliquots of medium were subjected to immunoprecipitation and electrophoresed as described in Materials and Methods. Cultures were treated with nothing or TGF-β (2 ng/ml) for the intervals indicated. (bottom panel) Densitometric analysis of the 50-kd region of the gel. Cultures were derived from a patient with severe Graves' ophthalmopathy.

4 hours approximately 50% of the band intensity was lost. At 16 hours of chase incubation, band intensity was identical to that found in the control cultures. There appeared to be approximately the same rate of loss of band intensity in the control cultures. The detectable level of PAI-1 in those cultures not receiving TGF-β was the consequence of a medium change. We have found that fresh serum upregulates PAI-1 expression in human fibroblasts (unpublished observations, 1994).

TGF-β Treatment of Orbital Fibroblasts Results in a Marked Increase in the Steady State Concentration of PAI-1 mRNA

We assessed the ability of TGF-β to alter the steady state levels of mRNA encoding for PAI-1 in orbital fibroblasts. When total cellular RNA extracted from confluent cultures is subjected to hybridization with a [32P]PAI-1 cDNA probe, two distinct transcripts, the products of alternative splicing, are expressed. The addition of TGF-β (2 ng/ml) to the culture medium for the times indicated in Figure 6 results in a substantial upregulation in the steady state levels of both 2.2- and 3.0-kb species in orbital cultures. As the figure demonstrates clearly, under control conditions, extremely low levels of PAI-1 transcript are expressed. By 4 hours of treatment with TGF-β, there is a profound induction of both transcripts (approximately 40-fold). The induction is maximal at 8 hours, when the increase is 60-fold above basal levels. By 16 hours, the levels of both transcripts begin to drop. These results are consistent with effects of TGF-β on PAI-1 being mediated at the pretranslational level. The lag between the peak of mRNA induction (8 hours) and that of mature protein (16 to 24 hours) suggests that an increased accumulation of translatable mRNA leads to an increased level of PAI-1 protein synthesis.

TGF-β Induces PAI-1 Expression in Orbital Fibroblasts From Normal Connective Tissue

To begin to determine whether the effects of TGF-β on PAI-1 expression in orbital fibroblasts was present in cultures derived from normal tissue as well as from patients with Graves' ophthalmopathy, multiple strains were studied in a single experiment. As the autoradiogram in Figure 7 suggests, there is little ECM-associated PAI-1 expressed constitutively in either of the two normal orbital fibroblast strains or the two from patients with the disease. The addition of the cytokine (2 ng/ml) resulted in a similar PAI-1 induction in all four strains tested. Three dermal strains exhibited substantial basal PAI-1 expression and a considerably less dramatic induction with TGF-β treatment. All three derive from normal-appearing skin, two from the abdominal wall and one from the shin. We also examined PAI-1 induction in three
strains of perimysial fibroblasts and found that TGF-β increased levels by an average of 7.3-fold.

**DISCUSSION**

Orbital fibroblasts exhibit intrinsic phenotypic attributes that set them apart from other types of human fibroblasts. From these studies, it is evident that PAI-1 in orbital cultures is under multicytokine upregulatory control and that TGF-β can induce substantially the protein in all strains thus far tested. We have reported that interferon-γ can also induce PAI-1 in fibroblast cultures while downregulating the protein or only modestly increasing its synthetic rate in dermal fibroblasts. Recent evidence has been advanced for tissue specificity in regard to the regulation by cytokines of PAI-1 in vivo. In that study, using a murine model, tissues responded differentially to endotoxin, TNF-α, and TGF-β. In the current studies, TGF-β upregulates PAI-1 synthesis in dermal and orbital cultures; however, the magnitude of response is substantially greater in orbital cultures. The relatively high basal expression in the dermal fibroblasts results in a relatively modest fractional increase despite the similar levels of PAI-1 expression achieved in both types of cytokine-treated cultures. It is the large magnitude of change in PAI-1 expression in orbital cultures exposed to TGF-β that implies the potential for substantial impact on ECM turnover in that cell type.

Our finding that orbital fibroblasts do not express constitutively high levels of PAI-1 has implications concerning inflammatory diseases of the orbit. It suggests that the economy of the ECM is ordinarily predicated on factors other than this polypeptide. Because PAI-1 can influence indirectly the activation of several metalloproteinases, the degradation rate of many molecular components of the ECM might be increased by PAI-1 induction. The hallmark of Graves' ophthalmopathy is the accumulation of hyaluronan and other ECM components. We have suggested that the cytokine-dependent increase in hyaluronan accumulation is a consequence of increased synthesis of the glycosaminoglycan in orbital fibroblasts. It is unknown whether the accumulation of the other poorly defined material is the consequence of increased rates of biosynthesis, decreased molecular turnover, or both. Another inflammatory process in the orbit resulting in the accumulation of extracellular matrix molecules is idiopathic orbital inflammation, or pseudotumor. In that condition, collagen deposition in the context of infiltration by immunocompetent cells predominate. Because PAI-1 is highly inducible in orbital connective tissue, altered rates of
Transforming growth factor-β has been shown to regulate the synthesis of a number of constituents of the ECM in several cell types. Types I, II, and VII collagen are all upregulated, as is fibronectin gene expression. In regard to PAI-1, TGF-β can upregulate the polypeptide in an established lung fibroblast line, and a putative response element for the cytokine has been identified in the 5' flanking region of the PAI-1 gene. Moreover, proteoglycan production is upregulated by TGF-β in dermal fibroblasts, and we have observed that the cytokine can increase the accumulation of glycosaminoglycan in orbital and dermal fibroblast cultures (unpublished observation, 1994). Thus, there is reason to implicate the cytokine as a potentially important determinant of the economy of the ECM in orbital connective tissue. Transforming growth factor-β has been found to stimulate macromolecular disposal remain potential explanations for much of the histopathologic picture associated with both Graves' ophthalmopathy and idiopathic inflammation.

Another feature of the histopathology of Graves' ophthalmopathy is the presence of activated T lymphocytes. The recruitment of these and other immunologically competent cells to the orbit suggests the possible roles of numerous soluble molecular factors in the pathogenesis of the disease. Human fibroblasts synthesize and release growth factors and cytokines, including TGF-β, interleukins-1, 8, and 11, and interferon α/β. Thus, further studies are warranted to define the peculiarities of the cytokine-elaborating repertoire of orbital fibroblasts, especially those derived from patients with ophthalmopathy.
the rates of orbital fibroblast proliferation. Moreover, the effects on proliferation were greater in cultures derived from patients with Graves’ ophthalmopathy than from normal orbs. We have reported that TGF-β can attenuate the induction by interferon-γ of major histocompatibility complex HLA-DR in orbital fibroblasts.

The molecular basis for the induction of PAI-1 by TGF-β in human fibroblasts is incompletely understood. Steady state PAI-1 mRNA levels are increased profoundly after treatment with the cytokine; however, we do not know whether gene transcription or increased transcript stability is responsible for these changes. Although these studies define another potential modulator of the pericellular proteolytic environment in orbital connective tissue, the role of TGF-β in its normal function or the pathogenesis of orbital disease remains to be defined. The magnitude of the PAI-1 induction in the orbital fibroblasts does strongly suggest that TGF-β can influence dramatically the pericellular environment of these cells in states of inflammation.

Key Words

serine protease inhibitor, cytokine, inflammation, Graves’ ophthalmopathy, extracellular matrix

References


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