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TRANSFORMING GROWTH FACTOR-β UP-REGULATES HUMAN ELASTIN PROMOTER ACTIVITY IN TRANSGENIC MICE

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We have recently developed transgenic mice which express ~5.2 kb of the human elastin promoter linked to the chloramphenicol acetyl transferase (CAT) reporter gene (*J. Biol. Chem.* 269:18072-18075, 1994). Previously, transforming growth factor-β (TGF-β) has been shown to enhance elastin gene expression, as determined at the mRNA and protein levels. To examine whether this enhancement could be explained by upregulation of the elastin promoter, TGF-β1 (100 ng) was injected subcutaneously into the transgenic animals. CAT activity in the skin of treated animals was elevated in a time-dependent manner up to ~10-fold after a single injection. These results suggest that the 5.2-kb up-stream segment of the human elastin gene contains *cis*-elements responsive to TGF-β1 *in vivo*.

Elastin, the major component of elastic fibers, is a well characterized connective tissue protein, which is initially synthesized as a ~70 kDa polypeptide encoded by a 3.5 kb mRNA (1-5). Expression of the elastin gene has been previously demonstrated by a variety of cell types, including aortic smooth muscle cells and dermal fibroblasts in culture (4-6). The expression of the elastin gene in such cells in culture has been shown to be modulated by a variety of factors, including transforming growth factor-β (TGF-β), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), insulin-like growth factor-1, various glucocorticosteroids, vitamin D_3, and cyclic nucleotides (7-15). Regulation of elastin gene expression by these affector molecules has been suggested to occur both at the transcriptional and post-transcriptional level.

Previously, TGF-β was shown to enhance elastin gene expression in porcine aortic smooth muscle cells, as determined at the protein level by using an ELISA (7). Subsequent studies demonstrated that treatment of human skin fibroblasts in culture with TGF-β elevated the elastin mRNA steady-state

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levels in a dose- and time-dependent manner (8). In the latter study, additional experiments utilizing transient cell transfections in fibroblast cultures with elastin promoter/CAT reporter gene constructs demonstrated no effect on elastin promoter activity, suggesting that the TGF-β effect was post-transcriptional (8). Furthermore, mRNA stability assays indicated that the half-life of elastin mRNAs was prolonged in the presence of TGF-β, again suggesting that stabilization of the transcript may result in elevated steady-state mRNA levels in culture (8).

In order to examine the transcriptional regulation of elastin gene expression in detail *in vivo*, we have developed a homozygous line of transgenic mice which provides a means to study the activity of the elastin promoter by utilizing a reporter gene, chloramphenicol acetyl transferase (CAT) (16). This animal model has been characterized with respect to tissue-specific and developmentally regulated expression of the elastin promoter (16). These transgenic animals were used to study the regulation of human elastin promoter activity by TGF-β1.

MATERIALS AND METHODS

Transgenic Mice Expressing the Human Elastin Promoter and CAT Assay. To examine the promoter-dependent control of the human elastin gene expression, we have recently developed a homozygous transgenic mouse line that expresses 5.2-kb human elastin promoter in a tissue-specific and developmentally regulated manner, as determined by assay of CAT reporter gene (16-18). These animals were used to study the effects of TGF-β by subcutaneous injections. CAT activity was then determined in the skin at varying time points. For this purpose, the skin specimens were excised from the site of the injections and homogenized in 0.25 M Tris-HCl, pH 7.5, using a Polytron tissue homogenizer. The homogenates were then centrifuged at 10,000 x g for 15 min at 4°C. The protein concentration of the supernatant was determined by a commercial protein assay kit (BioRad).

For CAT activity determination, 100 μg of protein was used for assay with [^{14}C]chloramphenicol as a substrate, as described elsewhere (18-20). The acetylated and non-acetylated forms of radioactive chloramphenicol were separated by thin layer chromatography, and CAT activity was determined by the radioactivity in the acetylated forms as a percent of the total radioactivity in each sample.

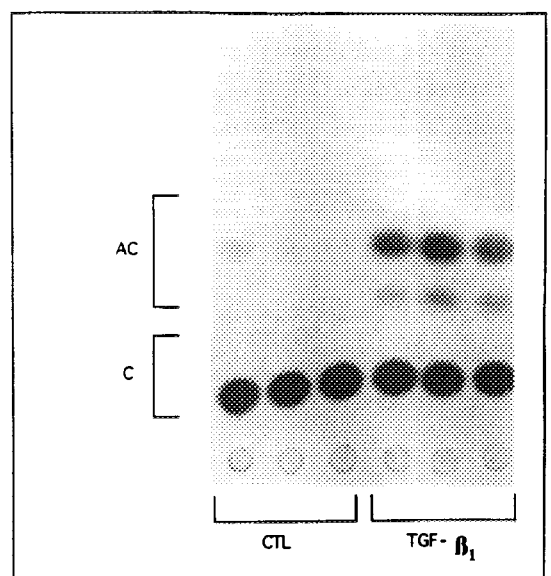
Cell Cultures. Smooth muscle cell and fibroblast cultures were established by the explantation method from aortae and skin of the transgenic animals, respectively (16). Fibroblast cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 5% newborn calf serum, 2 mM glutamine, and antibiotics. Smooth muscle cell cultures were maintained in M-199 medium containing the same supplements as above. The cell cultures were passaged by trypsinization and subjected to study at passages 2 or 3.

TGF-β Preparations. Human recombinant TGF-β1 was kindly provided by Dr. David R. Olsen, Celtrix Laboratories, Santa Clara, CA. For *in vivo* studies, 100 ng of TGF-β was injected subcutaneously in a total volume of 200 μl of normal saline, and the control animals received injections of saline alone. In cell culture studies, TGF-β was dissolved in the culture medium without fetal calf serum in a final concentration of 10 ng/ml. The incubations were continued for 24 hrs, and CAT assays were performed with cell extracts, as described above.

RESULTS AND DISCUSSION

Previous studies have demonstrated that TGF-β enhances the synthesis of elastin, as determined at the protein level by immunodetection techniques (7). Furthermore, incubation of human skin fibroblasts with TGF-β1 or TGF-β2 in concentrations varying from 1-5 ng/ml significantly increased elastin mRNA steady-state levels in a dose-dependent manner (8).

The transgenic mice developed as a model to study transcriptional regulation of the human elastin promoter (16) were used to examine the mechanisms of TGF-β up-regulation of elastin gene expression. For this purpose, TGF-β1 was injected subcutaneously into 5-day-old mice. The treated area was biopsied at different time points, up to 72 hrs, after the initial injection. Assay of CAT activity in the TGF-β1 treated mice demonstrated a marked increase, up to ~10-fold, in elastin promoter activity (Fig. 1).



	CTL	TGF-β_1
% of acetylation	3.40	36.22
Relative activity	1.0	10.65

Figure 1. Demonstration that TGF-β1 increases human elastin promoter activity in the skin of transgenic mice, *as determined by the* CAT assay. A single dose of TGF-β1 (100 ng) was injected subcutaneously in a total volume of 200 μl of saline, and the control animals (CTL) received the same volume of saline alone. After 24 hrs, a full-thickness 6-mm biopsy of the skin at the site of injection was removed, and CAT activity was determined, as described in Methods. The percent of acetylation of [^{14}C]chloramphenicol is expressed as the mean of 3 individual animals injected in parallel in both groups, each animal representing the same litter of transgenic mice. Calculation of the percent of acetylation indicated that TGF-β1 increased the human elastin promoter activity by ~10-fold.

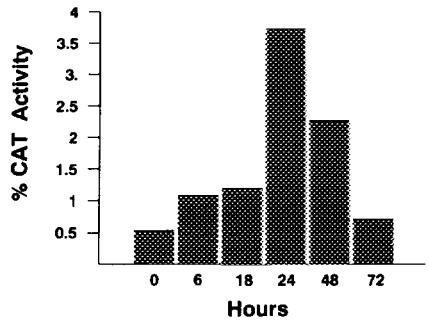


Figure 2. Time-course experiment of the effect of TGF-β1 on CAT activity in the skin of transgenic mice. The experimental design was the same as described in Figure 1, and biopsies of skin were obtained at the time points indicated following a single injection of TGF-β1. The values are the means of three separate experiments utilizing 4 different litters of mice, and each time point represents separate determinations from 4-6 individual animals.

Comparison of CAT activity at different time points after the injection indicated that the maximum activity was at 24 hrs, the values leveling off between 24 and 48 hrs, and significantly decreasing at the 72-hr time point (Fig. 2). Therefore, these observations suggested that TGF-β1 is capable of up-regulating human elastin promoter activity in the skin *in vivo*.

Previous studies utilizing transient transfections of human skin fibroblasts culture failed to demonstrate an effect of TGF-β1 on elastin promoter activity (8). Based on this observation, dermal fibroblast cultures from the transgenic animals were established, incubated with TGF-β1 (10 ng/ml), and CAT activity was determined after 24 hrs of incubation. Incubation of cultured skin fibroblasts with TGF-β1 did not affect the CAT activity, as compared with controls (Table I), confirming the previous

TABLE I. Effects of TGF-β1 on Human Elastin Promoter Activity in Aortic Smooth Muscle Cell and Skin Fibroblast Cultures Established from Transgenic Mice[a)]

Treatment	Smooth Muscle Cells[b)]	Fibroblasts[b)]
Controls	5.3 (1.0)	3.01 (1.0)
TGF-β	22.5 (4.2)	3.35 (1.1)

[a)]Cell cultures were established from transgenic animals, as described in Methods, and incubated with TGF-β1 (10 ng/ml) for 24 hrs. CAT activity in the cells was determined, as shown in Figure 1.

[b)]The values were expressed as percent acetylation of [^{14}C]chloramphenicol used as substrate for CAT assay and are means of 2 and 10 separate experiments with smooth muscle cell and fibroblast cultures, respectively, each assay being performed on duplicate cultures. The values in parentheses indicate the -fold enhancement of CAT activity in TGF-β1 treatedcultures in comparison to control cultures (1.0) incubated in parallel without TGF-β.

demonstrations using transient transfections of human skin fibroblasts (8). In contrast, smooth muscle cell cultures established from the aortae of the same transgenic animals clearly responded to TGF-β1, and CAT activities in two separate experiments were elevated 4.2-fold on the average, in comparison to control cultures incubated in parallel without TGF-β1 (Table I). Thus, there appears to be a cell-specific enhancement of elastin promoter activity in smooth muscle cells. Similar conclusions were recently reached in a study utilizing chick embryo aorta cells and tendon fibroblasts in transient transfections with human elastin promoter-CAT constructs (21). Specifically, incubation of transfected aorta cells with TGF-β resulted in a 2- to 4-fold increase in CAT activity, and the region from -196 to -12 of the promoter was suggested to play a major role in TGF-β induction (21). In contrast, no stimulation of promoter activity by TGF-β was noted in tendon fibroblast cultures. The latter cells were shown to be responsive to TGF-β with respect to fibronectin production (21). Thus, the aortic smooth muscle cells depict a cell-specific up-regulation of elastin promoter activity *in vitro*. The reasons for this cell-selective effect by TGF-β are not clear, but could suggest that specific *trans*-acting factors or selective signal transduction pathways may be operative in smooth muscle cells allowing up-regulation of elastin promoter activity by TGF-β.

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