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IGF-I Stimulates Tropoelastin Synthesis in Neonatal Rat Pulmonary Fibroblasts

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ABSTRACT. We have examined the effect of IGF-I on tropoelastin (TE) synthesis in cultured rat neonatal pulmonary fibroblasts, because this growth factor has been shown to stimulate TE synthesis in vascular smooth muscle cells. IGF-I stimulated TE and total protein synthesis in a dose-dependent manner even when cells were cultured in the medium supplemented with 0.5% FCS. The maximal stimulation was at IGF-I concentration 500 ng/mL and was an increase of 86 ± 14 and 35 ± 5% for TE and estimated total protein synthesis, respectively. There was a corresponding 95 ± 20% increase in the TE mRNA/β-actin mRNA ratio assessed by densitometry of the Northern blot analysis. At this low concentration of FCS, however, there was neither TE stimulation by dexamethasone alone nor in combination with IGF-I. We conclude that IGF-I stimulation of TE synthesis may occur in cells other than vascular smooth muscle cells and that there is no additive stimulation by glucocorticoids. (*Pediatr Res* 30: 248–251, 1991)

Abbreviations

RPF, rat pulmonary fibroblasts
TE, tropoelastin
DEX, dexamethasone

An extracellular protein component, elastin, may play an important role in the formation of the terminal airsac/alveolus during perinatal lung morphogenesis in various animals and in humans (1–8).

The pro-form protein, TE, is a soluble protein of approximately 72 000 to 75 000 molecular weight (9, 10), and the regulation of synthesis is poorly understood, partly because regulatory elements of the gene have not been fully defined (11). We have previously demonstrated that cultured RPF are elastogenic in early passaged cells and that TE expression is stimulated by DEX (12). To further understand the humoral factors that could stimulate TE synthesis in the developing lung, we have examined IGF-I in the present study. IGF-I has been shown to stimulate TE expression in vascular smooth muscle tissue and cells (13, 14), but this effect on other cell types including RPF has not been reported. We found that IGF-I stimulates TE synthesis in the neonatal RPF in a dose-dependent manner.

MATERIALS AND METHODS

Cell culture. Postnatal rats at 7 d were killed with an intraperitoneal injection of Nembutal (50 mg/kg; Eli Lilly Co., Indianapolis, IN). The project was approved by the Animal Care Committee of St. Louis University. Postnatal d 1 was counted as the birth date, and RPF were isolated as described before (12). Selection of the ages and response to DEX stimulation was dependent on our previous finding that TE expression is maximum at 7 d (12). Pups from two or three litters were combined and were used for extraction of total RNA and isolation of pulmonary fibroblasts by the differential adhesion method (15). The cells were passaged once and maintained in RPMI 1640 supplemented with 10% FCS until just before confluency and then in RPMI 1640 with 0.5% FCS to minimize the IGF-I effects of the FCS (14). At this FCS concentration, the results were more consistent than with 0.1% FCS. Twelve h after the medium switch, cells were treated with increasing concentrations of IGF-I for another 48 h, harvested by 0.25% trypsin and 0.01% EDTA treatment, and processed for extraction of total RNA (12). Cell counts were determined simultaneously using a hematocytometer. TE concentrations in conditioned media were determined by an ELISA.

Electron microscopy. A separate set of RPF at the first passage were grown in a Thermanox plastic dish (ICN Biomedical Inc., Costa Mesa, CA) in RPMI 1640 supplemented with 10% FCS. At 10 d (preconfluent) the RPF were fixed with 2% glutaraldehyde for 2 h at 24°C and embedded in Spurr's resin (Polysciences, Warrington, PA) and thin sections were examined with a model 100S electron microscope (JEOL USA, Peabody, MA).

Immunofluorescent staining. A fraction of RPF was passaged onto sterile microscopic slides and grown to preconfluency, and the cells were fixed with methanol at −20°C for 5 min and reacted with antibodies for vimentin, smooth muscle specific

Fig. 1. Representative transmission electron micrograph of a pulmonary fibroblast. The cells at the first passage were maintained for 10 d. Prominent cisternae of rough endoplasmic reticulum (*arrows*), a small amount of microfilamentous bundles (*arrowheads*), and the lack of an incomplete basal lamina outside the plasma membrane are characteristics of these cells. The cells often have lipid vacuoles (*L*) of different sizes, as seen in the *upper cell.* Magnification ×7800.

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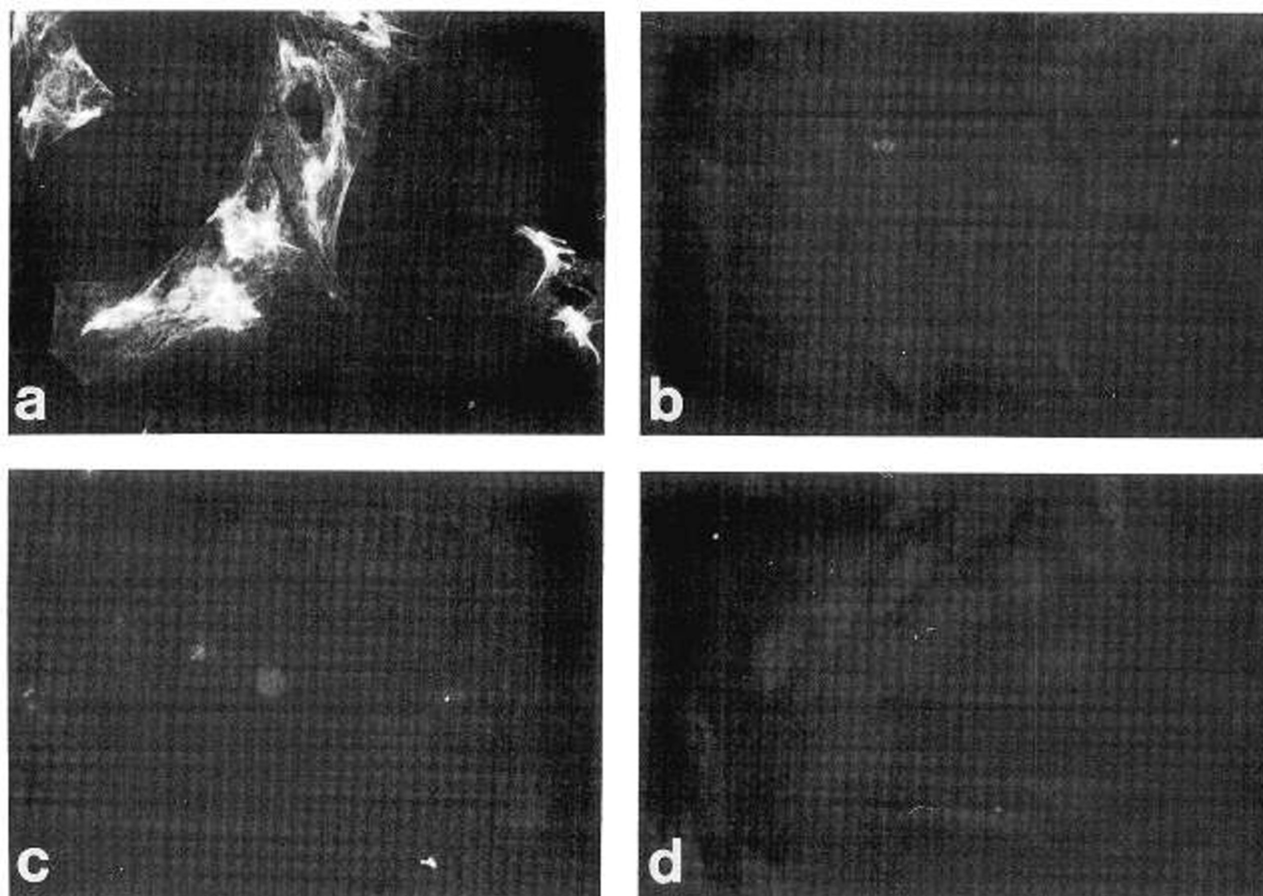

Fig. 2. Immunofluorescence for cytoskeletal proteins. Seven-d RPF were passaged once and fixed in methanol as described in Materials and Methods. The cells were positive for vimentin only. *a*, Vimentin; *b*, smooth muscle actin; *c*, smooth muscle myosin; *d*, desmin. Magnification ×400.

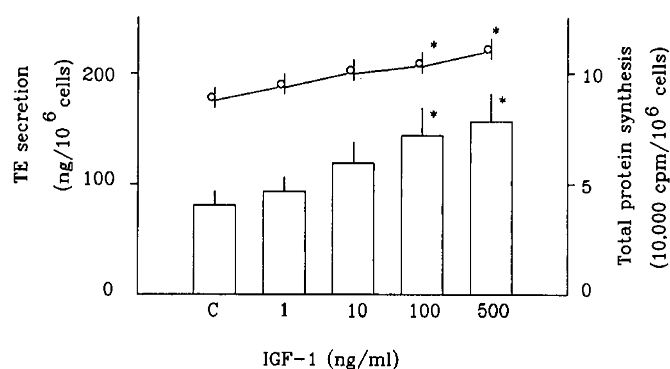

Fig. 3. Dose response of TE (*bar graph*) and ^{3}H-leucine incorporation into proteins (*line graph*) synthesis to increasing IGF-I concentrations. Secreted TE values were determined using the conditioned media, and protein synthesis values were determined using the media and cell extract combined sample as in Materials and Methods. Mean ± SEM of six different sample determinations. *, $p < 0.05$.

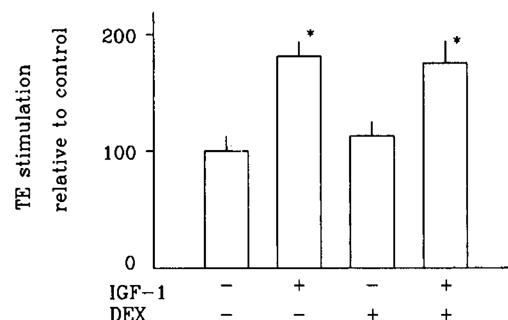

Fig. 4. TE stimulation examined in the presence of IGF-I (500 ng/mL) and/or DEX (10^{-7} M). Values were expressed as the percentage of increase from the control (100%). Mean ± SEM of six different sample determinations. *, $p < 0.01$.

actin, smooth muscle/skeletal muscle myosin, and desmin. The slides were then reacted with a fluorescein-conjugated second antibody and examined under a fluorescent microscope.

Total protein synthesis. Protein synthesis was estimated by determining ^{3}H-leucine incorporation into newly synthesized protein as described before (12) with minor modifications. The medium (5 mL/dish) and cell layer grown in a separate dish was washed three times with PBS, and media was switched to leucine-deficient RPMI supplemented with 0.5% FCS. RPF were treated with various concentrations of IGF-I for the last 48 h. At 12 h treatment time, 5 μL of ^{3}H-leucine (60 Ci/mmol, 1 mCi/mL) was added to the medium. Then the cell layer was scraped with a rubber policeman, and medium and cell layer together were collected in a tube and homogenized with a Tekmar (Cincinnati, OH) homogenizer for 10 s at 4°C. Aliquots of 100 μL were solubilized immediately in 0.1 N NaOH, neutralized in 0.1 N HCl, and precipitated with 200 μL of ice-cold 20% trichloroacetic acid, and the pellet was collected by centrifugation. After washing with ice-cold 10% trichloroacetic acid once, the pellet was dissolved in 100 μL of 1 M NaOH overnight at room temperature and counted in a Hewlett-Packard scintillation counter.

ELISA. TE concentration was determined by the method of ELISA as described before (12) using chicken anti-rat elastin antibody (1:2000), and rat α-elastin (40 ng/100 μL/well) was purified from rat aorta by the method of Partridge *et al.* (16) as the standard. TE content was calculated from the standard curve of the colorimetric reading using Minireader 2 (Dynatech,

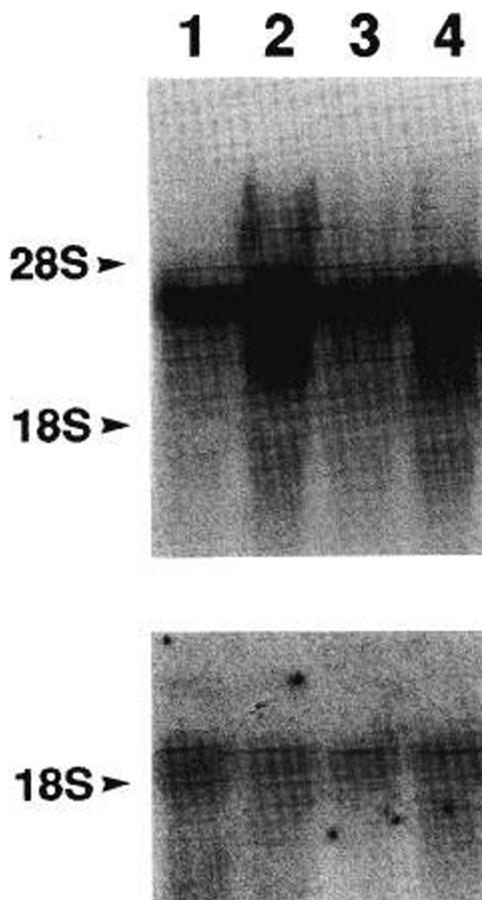

Fig. 5. Representative Northern blot analysis showing TE mRNA (*upper panel*) and β-actin mRNA (*lower panel*) in response to IGF-I (500 ng/mL) and/or DEX (10^{-7} M). *Lane 1*, untreated control; *lane 2*, IGF-I alone; *lane 3*, DEX alone; *lane 4*, IGF-I plus DEX.

Alexandria, VA). The background in unconditioned medium containing 0.5% FCS was subtracted from each value.

Northern blot analysis. The procedure was used as described before, except the amount of 20 μg per lane was fractionated by 1% agarose gel containing 6% formaldehyde. Hybridization was performed with nick-translated rat elastin cDNA ^{32}P-RE2 at 42°C in a buffer containing 50% formamide, 10 × sodium chloride sodium citrate buffer, 100 mM Na phosphate, pH 6.5, 10 × Denhardt's solution (17), and 1 mg/mL salmon sperm DNA. Washing conditions were as described before (12). The dried filter was exposed to Kodak XAR film at −70°C. Later, the same filter was hybridized with nick-translated chicken ^{32}P-β-actin cDNA (18) in pGEM 4Z and processed. β-Actin probe was used as the control probe to make a comparison with the TE mRNA. Densitometric analysis of the autoradiographs was performed using a model no. 620 video densitometer (Bio-Rad Laboratories, Anaheim, CA).

Materials. Chicken anti-rat α-elastin serum and RE2 cDNA were provided by Judith Foster, Ph.D. (Boston University) and used as described before (12). Recombinant human IGF-I was purchased from AmGen Biologicals (Oak Park, CA), and ^{32}P-nucleotide and ^{3}H-leucine were purchased from New England Nuclear (Boston, MA). Antibodies for vimentin, smooth muscle/skeletal muscle myosin, desmin, and fluorescein-conjugated antibody were from Sigma Chemical Co. (St. Louis, MO) and antibody for smooth muscle actin (CGA7) was from Enzo (New York, NY). All of the other chemicals were purchased from Sigma Chemical Co.

Statistical analysis. Analysis of variance was used to compare the different groups.

RESULTS

The fibroblastic characteristics of cells were examined to determine if the cells had any smooth muscle-like characteristics. Ultrastructurally, the cells have cytoplasmic lipid vacuoles, a small amount of microfilaments, and prominent cisternae of rough endoplasmic reticulum. Often, collagen fibers were recognized extracellularly. However, none of the cells at 10 d in culture had an incomplete basal lamina surrounding the plasma membrane as is characteristic of smooth muscle cells (Fig. 1). Immunostaining was positive for vimentin but negative for smooth muscle antigens: smooth muscle actin, smooth muscle myosin, and desmin (Fig. 2). Thus, ultrastructurally and immunohistochemically, the cells were fibroblasts and not differentiated smooth muscle cells. When these cells were treated with IGF-I, concentrations of TE in the cultured media increased in a dose-response manner and were 86 ± 14% greater than control at an IGF-I concentration of 500 ng/mL (Fig. 3). The ^{3}H-leucine incorporation increased progressively, and was 35 ± 5% greater than control at 500 ng/mL. Because DEX is known to stimulate TE synthesis in RPF (12), the effect of IGF-I plus DEX was studied. At this lower FCS concentration at 0.5%, DEX did not stimulate TE gene expression and did not increase the effects of IGF-I (Figs. 4 and 5). The TE mRNA in RPF also increased 95 ± 20% at an IGF-I concentration of 500 ng/mL (Fig. 5); the degree of stimulation was similar to the TE protein.

DISCUSSION

We have demonstrated that IGF-I is stimulatory for TE synthesis in neonatal RPF. The magnitude of stimulation by IGF-I was less than that reported in the vascular smooth muscle cells (14, 15). The IGF-I effect was preferential for TE as compared to that for total protein synthesis estimated by ^{3}H-leucine incorporation. Our results support the study by Foster *et al.* (19) on chick lung explant culture model, which showed that IGF stimulates TE synthesis in this system. However, the cell type responsible for the increased synthesis was not identified in that study. The present study confirmed that RPF are at least one of several responsive populations. Although the origin of the RPF is in question, our *in situ* hybridization studies have confirmed that the elastogenic fibroblasts in the 7-d rat lung are largely parenchymal rather than vascular adventitial or peribronchial (20). The cells examined were ultrastructurally shown to be fibroblastic and not differentiated smooth muscle cells. By immunofluorescence also, smooth muscle specific antigens were not seen in these cells. The common ultrastructural characteristics in all of the cells examined were prominent rough endoplasmic reticulum cisternae and lack of basal lamina around the plasma membranes. Cultured vascular smooth muscle cells change from a contractile to a synthetic phenotype in culture (21, 22), which includes development of prominent rough endoplasmic reticulum cisternae (22) and formation of an incomplete basal lamina within a few days. These characteristics distinguish smooth muscle cells from fibroblasts (22). Because cells were in culture for 10 d before fixation, a basal lamina should have been detected if they were in fact differentiated smooth muscle cells. Thus, the IGF-I effect on TE synthesis is not restricted to vascular smooth muscle cells, and it could possibly stimulate TE in the lung parenchyma *in vivo*. However, the results should be interpreted with caution because the maximal concentration to elicit a significant response was higher than the usually observed cellular response by IGF (23). The response was not mediated by insulin receptors because insulin stimulation of TE required an approximately 20 times greater molar concentration than IGF-I (unpublished observations). The fact that DEX with 0.5% FCS alone or in combination with IGF-I did not stimulate TE synthesis in this study contrasts with the results of our previous study using 10% FCS, when this was achieved (12). This suggests that DEX stimulation of TE requires a higher concentration of other factor(s) different from IGF-I that are present in FCS.

The physiologic role of IGF in the developmental lung is far from clear, and their specific effects on extracellular matrix formation in the lung are limited. IGF-I was shown to stimulate collagen in human lung fibroblasts *in vitro* (24). IGF-I is secreted by fetal rat lung fibroblasts and stimulates cell multiplication in an autocrine or paracrine fashion (25). The question of whether IGF-I plays a significant role in lung morphogenesis and/or in the disease states through modulation of extracellular matrices such as elastin and collagen remains to be answered.

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