We have reported previously that a factor released by elastase treatment of pulmonary fibroblast cultures is capable of down-regulating elastin gene expression. In the present study we have pursued the identification of the factor released by elastase treatment and the characterization of the level of elastin gene expression at which this factor exerts its effect. We have found by immunologic and biochemical procedures that elastase treatment results in the release of basic fibroblast growth factor (bFGF) that is bound within the matrix. Both purified bFGF and bFGF released by elastase from cell matrices decrease the transcriptional level of the elastin gene by 70–80% within 24 h. Transient transfections of pulmonary fibroblasts with a series of elastin promoter deletion constructs show that the region of the elastin gene responsive to bFGF is located within sequences spanning −900 to −200 base pairs. The biological implications of these findings coupled with our previous report are significant, since they demonstrate that elastase digestion of pulmonary fibroblast matrices not only results in the proteolysis of elastin but also results in the release of a potent regulator of elastin gene transcription whose activity can influence repair mechanisms.

Elastin is an extracellular protein whose intrinsic ability to passively expand and contract under gas and liquid pressure gradients renders it an important functional element in maintaining proper pulmonary function. The formation of insoluble elastin in the developing alveolar wall is an essential step in imparting the ability of the developing mammalian lung to meet the demands of gas exchange in respiratory dynamics. In pulmonary obstructive diseases such as emphysema, the continual loss of elastin from alveolar walls with concomitant enlargement of air spaces is a significant factor in the pathological process. This loss of elastin is thought to result from elastase activities arising from enzymes secreted by an influx of macrophages and neutrophils in the airways after prolonged physical or chemical insult (Albin et al., 1987; Sandhaus, 1987; Snider et al., 1991). Although metabolic labeling studies have shown that elastin can be resynthesized to pre-insult levels, ultrastructural evaluation suggests that the repair is disorganized and dysfunctional (Kuhn et al., 1976).

Previously we have reported on an in vitro model, which mimics conditions proposed to trigger the development and perpetuation of the emphysematous condition, i.e. elastase digestion of the extracellular matrix (Foster et al., 1990). In this model primary cultures of neonatal rat pulmonary fibroblasts, which synthesize a matrix containing insoluble elastin as well as other essential matrix components, are briefly exposed to elastase and the cellular response examined. Using this system we have found that the products released by elastase exposure are capable of significantly down-regulating steady-state levels of elastin mRNA in the control pulmonary fibroblast cultures. Since the amino acid analysis of the elastase-solubilized fraction exhibited a composition indicative of elastin, we hypothesized that elastin peptides were capable of autoregulating elastin gene expression in a negative manner (Foster et al., 1990).

The overall goals of the present study were to determine the level at which elastase solubilized products of pulmonary fibroblast cultures down-regulate elastin mRNA and to identify the elastin peptide responsible for this activity. We have found that the down-regulation of elastin expression occurs at the transcriptional level, and, contrary to our initial hypothesis, the factor responsible is not an elastin peptide but rather bFGF, which is released from the matrix upon elastase treatment.

**MATERIALS AND METHODS**

*Reagents—* Human recombinant bFGF (18 kDa) was from Scios-Nova (Mountain View, CA). Mouse monoclonal anti-bovine bFGF, Type II IgG (Upstate Biotechnology Inc., Lake Placid, NY), was used for immunocytochemistry and Western blot analyses. Mouse monoclonal anti-bovine bFGF, Type I IgG (Upstate Biotechnology Inc.), was used to neutralize bFGF activity.

*Establishment and Treatment of Cell Cultures—* Neonatal rat pulmonary fibroblast cells were isolated from lungs of 2–3-day-old Sprague-Dawley rats and seeded in first passage in 75-cm² flasks at a density of 2 × 10⁴ cells/cm² in 20 ml of Dulbecco’s modified Eagle’s medium (DMEM) and 5% fetal bovine serum (FBS) as described previously (Foster et al., 1990). Medium was changed twice weekly. After 18 days, the cell cultures were treated with either elastase-generated digest (5–15 μg/ml) or purified bFGF (2–50 ng/ml) for 24 h.

The generation of elastase matrix products was performed essentially as we have previously detailed (Foster et al., 1990) but with several differences. After collection of the medium from elastase digestion and treatment to inhibit elastase, the mixture was either used directly or was lyophilized, dissolved in 0.1 original volume of 44 mM NaHCO₃ and then diazylated against 44 mM NaHCO₃ using a membrane

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1 The abbreviations used are: bFGF, basic fibroblast growth factor; Ab, antibody; bp, base pair(s); BSA, bovine serum albumin; CAT, chloramphenicol acetyl-CoA transferase; CLSM, confocal laser scanning microscopy; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GFM, fibroblast growth factor; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TGF-β, transforming growth factor-β.
with a molecular weight exclusion of 6,000. The protein concentrations of the original and concentrated digest were determined by the BCA protein assay (Pierce).

The Type I neutralizing monoclonal antibody to bFGF (Upstate Biotechnology Inc.) was used to inhibit the activity of bFGF in the cell cultures. Specified amounts of antibody were added to the cell cultures at the same time as 10 ng/ml bFGF or 5 μg/ml elastase-generated digest and the cells incubated for 24 h.

**Immunocytochemistry and Confocal Analysis—**Pulmonary fibroblasts were cultured on dual chamber glass slides (Nunc) for 10 or 14 days. Cells were treated with elastase (0.5 μg/ml) or with buffer alone for 10 min at 37 °C and then washed once with PBS and fixed with 3.7% formaldehyde for 30 min at room temperature. Fixed cultures were washed twice with PBS and incubated in blocking buffer (PBS, 3% BSA, 0.1% NaN3) at room temperature for 1 h. The cultures were then exposed to x-ray film at −80 °C for 5 days and then analyzed on a phosphorimager cassette (24–48 h) for quantification using a Molecular Dynamics Phosphorimager.

**Transcription Nuclear Run-on Analysis—**Nuclei from control, elastase-generated digest (5 μg/ml), and bFGF-treated (10 ng/ml) pulmonary fibroblast cultures were isolated and subjected to nuclear run-on analyses as described elsewhere (Wolfe et al., 1993). Two separate sets of primary cell cultures were used for these analyses with each set run in duplicate. Ten micrograms of pBluescript DNA and chimeric pBluescript DNA containing cDNA for trolopelastin, lysyl oxidase (gift of Dr. Philip Trackman), actin, and histone 2B were applied to nitrocellulose using a slot blot apparatus. The prehybridized filters were incubated with recovered run-on solutions (4 × 10⁶ cpm/ml) at 65 °C for 72 h, then washed and treated with RNase (Wolfe et al., 1993). The nitrocellulose filters were then first exposed to x-ray film at −80 °C for 5 days and then analyzed on a phosphorimager cassette (24–48 h) for quantification using a Molecular Dynamics Phosphorimager.

**Western Blot Analysis—**Digests recovered from elastase- and mock-treated pulmonary fibroblasts were made 1 μg in diisopropyl fluorophos- phate to inhibit elastase activity and then directly analyzed by 18% SDS-PAGE. Recombinant bFGF and a set of protein molecular weight standards were included on each gel analysis. The samples were elec-etrophoretically transferred to nitrocellulose by the method of Towbin et al. (1979). The blot was treated as we have described previously (Jensen et al., 1995) using the Type II monoclonal antibody to bFGF (1 μg/ml) and goat anti-mouse IgG (Bio-Rad) conjugated to horseradish peroxi- dase and then treated with Amersham ECL reagents and exposed to x-ray film for specified times.

**125I-bFGF Binding and Release—**Equilibrium binding of 125I-bFGF was conducted with confluent pulmonary fibroblasts (Nugent and Edelman, 1992). 125I-bFGF was prepared using a modification of the Bolton-Hunter method that we have described previously (Nugent and Edelman, 1992). Single monolayer cultures (5 × 10⁵ cells/well) were plated in 24-well plates (2 cm²/well, Costar, Cambridge, MA) in 1 ml of DMEM, 10% FBS and incubated at 37 °C for 2 days. Prior to initiating the binding assay, the culture medium was removed and the cells were washed once with binding buffer (DMEM, 25 mM HEPES, 0.05% gelatin) at 4 °C. Fresh binding buffer was added (0.5 ml/well), and the cells were incubated at 4 °C for 10 min. 125I-bFGF (20 ng/ml) was added, and the cells were incubated at 4 °C for 2.5 h. At the end of the binding incubation, non-bound 125I-bFGF was removed by washing the cells four times: three times with cold binding buffer followed by one wash with PBS at room temperature. The cells were then incubated with 44 mM NaHCO₃, pH 7.4, with and without elastase (0.5 μg/ml) for various times (0–30 min) at room temperature. At each time point the enzyme solution was removed, the cells were washed once with PBS and extracted with 1 N NaOH, and the amount of cell-bound 125I-bFGF was quantitated with a γ counter. The amount of 125I-bFGF bound to the cells at each time point was subtracted from the amount bound at time zero (1.06 ± 0.06 ng/well) to determine the amount released. Observed first order rates were determined by non-linear least squares curve fitting (Kaleida-Graph version 3.0.4, Synergy Software, Reading, PA).

**Isolation and Analysis of RNA—**Total RNA was isolated and ana-lyzed by Northern blotting as described previously (Wolfe et al., 1993). Duplicate 10-μg samples of total RNA were fractionated on a 1.1% agarose, 6% formaldehyde gel and electrophoretically transferred to a Nytran filter (Foster et al., 1988). Prior to electrophoretic transfer, the gel was cut in half so that one of each duplicate sample could be transferred with acidinine orange to monitor sample loading and RNA integrity and to establish electrophoretic size markers. After electrophoretic transfer of the gel to Nytran, hybridization was performed with 32P-labeled rat trolopelastin (Rich and Foster, 1989) and rat actin (gift of Dr. Nadal-Ginard) cDNAs as described previously (Wu et al., 1992). The filter was first exposed to x-ray film at −80 °C using an intensifying screen and then analyzed on a phosphorimager cassette for quantitation using a Molecular Dynamics Phosphorimager.

**RESULTS**

**Fibroblast Growth Factor Mimics the Action of Matrix Degradation Products on Elastin mRNA Levels—**We have reported previously that the addition of elastase-generated, matrix degra-dation products to pulmonary fibroblasts cultures resulted in the down-regulation of elastin protein and mRNA levels. In attempts to purify the active agent in the elastase solubilized products, we initially focused our efforts on the possibility that the active compound is an elastin fragment released by elastase treatment. This hypothesis was based on the amino acid composition of the protein/peptide fraction released by elastase treatment, which exhibited a composition very similar to that of rat insoluble elastin. We found that the more we fractionated the digest, to purify elastin peptides, the less activity we recovered. These observations, together with our inability to demon-strate either reproducible or significant activity with different purified rat insoluble elastin digests and rat trolopelastin preparations, led us to explore the possibility that a low abun-dant but potent growth factor or cytokine was released from the matrix by elastase treatment. Two such factors, i.e. bFGF and TGF-β, are known to be associated with matrix molecules and both are capable of modulating elastin gene expression (Liu and Davidson, 1988; Kahari et al., 1992; Brettell and McGowan, 1994). Although TGF-β did not alter elastin mRNA levels (data not shown), bFGF had a significant effect. The Northern blot given in Fig. 1 compares the addition of elastase-generated peptides and purified bFGF on the steady-state lev-els of elastin and actin mRNAs. The addition of elastase digest to the pulmonary fibroblasts resulted in a 78 ± 10% reduction in elastin mRNA consistent with our previous findings (Foster et al., 1990). Interestingly, the addition of 10 ng/ml FGF also
decreased elastin mRNA levels to comparable levels in the three separate experiments, i.e. 80 ± 10%. The levels of actin mRNAs were not changed by either treatment, showing that the decrease in elastin mRNA levels is not indicative of a generalized response.

Elastase-generated Peptides and Basic Fibroblast Growth Factor Decrease Levels of Elastin Transcription—Nuclear run-on assays were performed on nuclei isolated from pulmonary fibroblasts cultures treated with either elastase-generated digest or bFGF. Fig. 2A provides the nuclear run-on blots obtained from a typical analysis performed, and Fig. 2B provides a quantitative analysis of data obtained from three separate sets of pulmonary fibroblasts run in duplicate. The transcriptional level determined for each gene in the control cultures is given as 100%, and the levels obtained after addition of either elastase digest or bFGF to the cells are plotted as a percent of that control. These results demonstrate that both the elastase-generated digest and bFGF down-regulate elastin transcriptional levels by approximately 83–87%, which is in very close agreement with the reduction of the steady-state levels of elastin mRNA. Transcription levels of lysyl oxidase and actin do not change significantly with either treatment. The transcriptional level of histone 2B remained relatively constant among control, elastase digest, and bFGF-treated cells, suggesting that neither treatment results in a general increase in the number of cells entering S phase. However, we did not render the cells quiescent or attempt to synchronize them in G0 so that we cannot exclude the possibility that either the digest or bFGF contributes to cell cycle progression within the limited 24-h period examined.

Basic Fibroblast Growth Factor Is Released after Treatment of the Pulmonary Fibroblasts Cultures with Elastase and Is the Active Factor in the Down-regulation of Elastin mRNA Levels—Since the effect of bFGF on elastin transcriptional and mRNA steady-state levels closely mirrored that of the elastase digest, we investigated the possibility that bFGF was both present in the matrix of the pulmonary fibroblast cultures and that it was released into the medium by elastase treatment. Several approaches were used to address these possibilities. Second passage fibroblast cells were plated onto dual chamber slides, allowed to grow for 10–14 days, and then processed for immunocytochemistry analysis. Photomicrographs of CLSM images were obtained from elastase and vehicle-treated pulmonary fibroblasts stained with a monoclonal antibody to bFGF are shown in Fig. 3. The images presented represent sections near the basal surface of the cells (6 μm down from the apical surface). Intracellular staining was apparent in both control and elastase-treated cultures. However, significant extracellular staining was only observed in control cultures, suggesting that elastase treat-
only. We do not know if this band is related to bFGF or simply interacts nonspecifically with the antibody used.

To further confirm that elastase can facilitate bFGF release from the pulmonary fibroblast matrix, the release of 125I-bFGF from fibroblast matrices was measured in the presence and absence of elastase. 125I-bFGF was incorporated into the matrices of pulmonary fibroblast by incubation of the cells with 20 ng/ml 125I-bFGF at 4°C. Under these conditions cellular internalization is inhibited and bFGF is predominantly bound to heparin sulfate within the matrix (95% of all bFGF bound) (Nugent and Edelman, 1992). The 125I-bFGF-labeled cultures were then incubated in buffer with or without elastase for various times. As shown in Fig. 5, the presence of elastase accelerated the rate of bFGF release from the matrix 22-fold (apparent first order rate constants, \( k_{\text{obs}} = 0.014 \text{ min}^{-1} \) for control; \( k_{\text{obs}} = 0.314 \text{ min}^{-1} \) for elastase-treated cells).

In order to test directly whether bFGF is the factor in the elastase digest that is responsible for the down-regulation of elastin mRNA, a neutralizing antibody to bFGF was added to fibroblast medium together with either the elastase-generated peptides or bFGF. Fig. 6A shows a Northern blot and Fig. 6B the quantitative analyses of three experiments performed with separate sets of primary fibroblasts. A preparation of nonimmune serum was used to establish nonspecific interactions. Although not shown, rRNA and actin mRNA were used to determine loading and specificity. Whereas the addition of 20 or 40 μl of antibody to cells simultaneously exposed to bFGF (10 ng) partially blocked elastin mRNA down-regulation, the addition of 40 μl of antibody together with the elastase digest not only blocked the down-regulation but also resulted in a small but reproducible increase in mRNA levels over control. Although not shown, the addition of 40 μl of antibody to control cultures also results in a 10% increase in elastin mRNA. These data directly link the active agent in the elastase digest to bFGF and further suggest that elastin gene expression may be negatively regulated by endogenous bFGF in the control pulmonary fibroblasts.

The FGF Response Element Is Located in the Distal Region of the Human Elastin Gene Promoter—Pulmonary fibroblast cells were transiently transfected with a series of human elastin promoter deletion constructs in the absence and presence of 10 ng/ml bFGF. A representative CAT assay for these elastin gene constructs in control (–) and bFGF-treated (10 ng/ml medium) cells (+) is provided in Fig. 7. The amount of extract from each transfection assayed for CAT activity was normalized to 100 μg...
of protein and adjusted for transfection efficiency by co-transfection and measurement of β-gal activity. The results of four separate transfection experiments using two different preparations of plasmid DNAs are given in Fig. 8. In the control fibroblast cultures, the elastin gene deletion constructs dictate a basal pattern of activity reflecting several functional regulations of plasmid DNAs are given in Fig. 8. In the control, the steady-state level of elastin mRNA in the control was set at 100% in each analysis, and the levels obtained after specified treatments were plotted as a percent of that value. Values represent the average of three separate sets of primary cultures.

FIG. 7. Transient expression of elastin/CAT chimeric constructs in control and bFGF-treated pulmonary fibroblasts cultures. Pulmonary fibroblast cell cultures were co-transfected with 30 μg of the various elastin/CAT gene constructs (size denoted in figure) and 5 μg of pCMV p-gal in the (+) presence (10 ng/ml of medium) or (−) absence of bFGF (see "Materials and Methods" for details). Aliquots of cell lysates, containing equal amounts of β-galactosidase activity, were subjected to thin layer chromatography in order to separate 14C-acetylated cholraoamphicol derivatives and the resultant thin layer plate exposed to x-ray film. A control lane (C) with CAT enzyme is also shown.

DISCUSSION

In a previous publication from our laboratory, we reported on the development of an in vitro model for examining mechanisms operative in the development and propagation of pulmonary emphysema. Experimental data obtained from that model showed that elastase digestion of pulmonary fibroblast matrices results in the release of a factor(s) that could influence elastin gene expression in a positive or negative fashion dependent upon whether the cultures had been treated with elastase prior to the addition of the elastase digest (Foster et al., 1990). In the present study, we have focused on the ability of the elastase digest to down-regulate elastin gene expression in control cell cultures (no elastase treatment) since this is the simpler of the two situations. Within this system, we initially pursued the hypothesis that an elastin fragment was the likely candidate to target within pulmonary fibroblast cells, especially since Brettell and McGowan (1994) have already shown that bFGF decreases elastin production in cell cultures similar to those affected by elastase digestion.
used in our studies. These latter investigators demonstrated that bFGF was capable of decreasing elastin mRNA and protein levels and suggested that regulation could be exerted at the transcriptional level based on transient transfections of a CAT reporter gene driven by a 2.26-kilobase human elastin gene promoter fragment. Additionally, Davidson et al. (1993) have shown previously that bFGF inhibits the TGF-β stimulation of elastin production in smooth muscle cells. In the present study, we have directly measured transcription levels of the elastin gene by nuclear run-on experiments and have found that both elastase digest and bFGF decrease elastin gene transcription levels by comparable and signficant amounts. More importantly, we have shown that bFGF is a factor released by elastase digestion of pulmonary fibroblast cell matrices thereby attributing its activity to the biologically relevant situation of injury/repair known to exist in the development and progression of pulmonary emphysema (Sandhaus, 1987; Snider et al., 1991).

Although we have shown that bFGF is a potent modulator of elastin gene transcription within an environment mimicking elastase-induced injury, we were unable to specifically localize the bFGF response region within the promoter except for excluding its existence within the proximal promoter region, i.e. −195 to +2. The magnitude of the effect found by transient transfections is not as great as that measured for the endogenous gene response. The reasons for our inability to localize more precisely the region of bFGF responsiveness and the magnitude of that response may reside in the fact that we are using a heterologous system consisting of human elastin promoter sequences within rat cells or may reflect the use of an inappropriate reporter activity (CAT) to investigate down-regulation. We will address both of these issues in future studies by using rat elastin gene promoter constructs driving a luciferase reporter. We do not plan to switch to stable transfections since we have already shown that creating stable transfectants by repeated passages of pulmonary fibroblasts results in a dramatic decrease in elastin synthesis as well as a complete loss of elastin gene regulation.2

The results obtained in this study are important to developing hypotheses for mechanisms underlying normal and abnormal repair of pulmonary elastin. Our previous work suggested that the ability of the fibroblast cells to synthesize tropoelastin after elastase digestion was dependent on both its location relative to the site of injury and its competency to respond to factors released by elastase digestion of matrix components. Since bFGF has been identified as one of the components released by elastase treatment of fibroblast cultures, this suggests that bFGF may act as a repressor of elastin gene transcription in cells remote from an elastase injury, perhaps to prevent a general fibrotic response. Cells adjacent to the elastase-induced injury might not be immediately affected by bFGF release due to damage to FGF receptor or FGF-binding proteoglycan. Additionally, the cells adjacent to elastase activity might be selectively more responsive to potential up-regulators of elastin as the result of differential proteolytic damage.

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