Heparin-binding EGF-like growth factor regulates elastin and FGF-2 expression in pulmonary fibroblasts

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Lung interstitial elastin plays a critical role in allowing air exchange through the alveolar network. This role is underlined in the development of chronic obstructive pulmonary disease (COPD) such as emphysema, where proteolytic degradation of elastin within alveolar walls is associated with the destruction of lung architecture and the concomitant loss of lung function. Elastin is synthesized as a soluble precursor, tropoelastin, that is secreted and cross-linked to form insoluble elastic fibers. In rodents, lung elastin gene expression is high during late fetal and early postnatal development, when the majority of elastin mRNA is localized in the pulmonary vasculature and later in the forming alveolar septa and crests (32). Elastin gene expression decreases afterwards, and there is very little elastin synthesis in adults. Temporal and spatial association of elastin synthesis with specific developmental events suggests that elastin plays an active role in lung morphogenesis. Indeed, elastin deposition within the tips of the secondary septa is thought to be a driving force for alveolarization in rodent postnatal lung development (3, 8, 27). In addition, elastin-deficient mice exhibit retarded lung branching morphology at birth, suggesting an earlier contribution of elastin in terminal airway branching (50).

A number of animal models of emphysema, based on administration of elastolytic proteases, have been used to analyze the biochemical changes accompanying the loss of elastin from lung parenchyma. In these animal models, the rapid decrease of lung insoluble elastin levels is followed by a reactivation of elastin gene expression (45). The observed elastin resynthesis following the proteolytic damage is thought to represent an attempt to repair the proteolytic injury. However, human emphysema is characterized as a chronic disease resulting from repeated exposure to pathological insults that are thought to eventually compromise an effective repair. Mechanisms underlying the regulation of elastin gene expression in development and injury/repair situations are not well understood, although they may share some common mediators. The complexity of lung architecture makes it difficult to perform biochemical analyses in vivo. The injury/repair process is further complicated by the involvement of accompanying inflammatory responses that include an influx of cells and their release of cytokines. Thus the use of in vitro cell culture models allows a simple system for dissection of the effects of various factors and their mechanism of action.

Primary cultures of rat pulmonary fibroblasts synthesize an insoluble elastic matrix similar to that
found in alveolar walls (5). Brief treatment of these cultured fibroblasts with pancreatic elastase solubilizes elastin (15). The resultant elastase digest exhibits an inhibitory activity on elastin gene expression when added exogenously to untreated fibroblasts (15). Previously, we have identified fibroblast growth factor-2 (FGF-2) as a major elastogenic inhibitory factor within the elastase digest (44). FGF-2 is a heparan sulfate proteoglycan (HSPG)-binding growth factor that is initially sequestered in the matrix of pulmonary fibroblasts. Elastase cleavage of the core proteins depletes cell-associated HSPG and mobilizes FGF-2 (4). In addition, elastate treatment of the cells decreases the equilibrium binding of FGF-2 to both HSPG and receptor sites, suggesting a modified FGF-2-dependent response in elastate-treated cells (4).

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is another HSPG-binding growth factor that has been implicated in lung pathogenesis induced by various factors (23, 38, 48, 53). HB-EGF is a potent mitogen for lung fibroblasts and epithelial cells (29, 53) and has been shown to stimulate cell migration in other cell systems (31, 47). HB-EGF is a member of the EGF family that consists of a number of other structurally related growth factors such as EGF, transforming growth factor (TGF)-α, and amphiregulin (40). Unlike EGF or TGF-α, HB-EGF possesses a heparin-binding domain (46) that allows its interaction with cell surface and matrix HSPG and subsequent modulation of its biological activity (18). EGF family members bind and activate the tyrosine kinase receptor epidermal growth factor receptor (EGFR) to regulate diverse processes in various cell types (39). Importantly, HB-EGF has been shown to decrease elastin gene expression in chick aortic smooth muscle cells (22) and lung fibroblasts (10). The effect of HB-EGF on elastin gene expression has not been examined. Because its activity is regulated by HSPG (18), and HSPG is a target of elastase treatment, HB-EGF may be an important signaling ligand in elastase-induced injury/repair.

The goal of this study is to investigate the potential role of HB-EGF in regulating elastin gene expression in pulmonary fibroblast cultures. We first established that elastase treatment of matrix-laden pulmonary fibroblast cultures resulted in a detectable amount of soluble HB-EGF within the elastase-released products. Addition of soluble HB-EGF to undigested fibroblast cultures decreased the steady-state level of elastin mRNA through a mechanism similar to that reported for FGF-2 (6, 42). Furthermore, we show that HB-EGF treatment of pulmonary fibroblasts leads to an increase in endogenous FGF-2 mRNA and protein levels.

MATERIALS AND METHODS

Reagents. Recombinant human HB-EGF and goat polyclonal antibody against human HB-EGF (cat. no. AF-259-NA) were obtained from R&D Systems (Minneapolis, MN). Recombinant human FGF-2 (18 kDa) was purchased from Scios-Nova (Mountain View, CA). AG-1478 was obtained from Calbiochem (La Jolla, CA). PD-98059 and U-0126 were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibodies against Fra-1 (cat. no. sc-183), c-Jun (cat. no. sc-45), horseradish peroxidase-conjugated goat anti-rabbit, donkey anti-goat, and goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-phospho-ERK1/2 (cat. no. 9101) was obtained from New England Biolabs (Beverly, MA). Rabbit polyclonal anti-rat ERK1/2 (cat. no. 06-182) and mouse monoclonal antibody against bovine FGF-2 (cat. no. 05-118) were purchased from Upstate Biotechnology (Lake Placid, NY).

Cell cultures and treatment. Neonatal rat pulmonary fibroblast cells were isolated from the lungs of 3-day-old Sprague-Dawley rats as described (15). For most experiments, second-passage fibroblasts were plated in 75-cm² flasks at 2 x 10⁴ cells/cm². In some experiments, cells were seeded in 35-mm plates at 10 x 10⁴ cells/dish. Cells used for ¹²⁵I-HB-EGF binding assays were plated at 5 x 10⁴/well into 24-well plates (2 cm²/well; Costar, Cambridge, MA). After being plated, cells were maintained in DMEM containing 5% FBS for 2 wk. Before elastase treatment, the confluent, matrix-laden cultures were rinsed twice with Puck’s saline (137 mM NaCl, 5.37 mM KCl, 1.10 mM KH₂PO₄, 1.08 mM Na₂HPO₄, 6.10 mM glucose, pH 7.4) and once with the digestion buffer (44 mM sodium bicarbonate, pH 7.4). Pancreatic elastase in sodium bicarbonate solution (5 µg/ml) was added to the cells, and the flasks were incubated at 37°C for 5 min. The control cultures were treated similarly except that they were incubated with sodium bicarbonate solution without the supplement of elastase. At the end of the 5-min period, digestion buffers covering the control and treated cells were collected, and diisopropyl fluorophosphate (DFP) was added at a final concentration of 1 µM to inhibit further elastase activity. Elastase digests were kept frozen in −80°C if not used immediately.

In experiments studying the effects of exogenously added HB-EGF and/or FGF-2, 2-wk-old fibroblasts were fed with fresh medium containing 0.5% FBS and were cultured for 24 h before ligand addition. Unless otherwise specified, 10 ng/ml of growth factors were added. In some experiments, cells were preincubated with either the inhibitors (AG-1478, 10 µM; PD-98059, 15–75 µM; U-0126, 25–75 µM) or an equal amount of solvent (DMSO or methanol) for 1 h before the addition of growth factors.

Analysis of elastase digest. The original elastase digests were concentrated to 1:20 of the original volume using the SPD1010 SpeedVac System (Thermo Savant, Holbrook, NY) and subjected to an SDS-PAGE clean up kit (Amer sham Biosciences, San Francisco, CA). The resultant material was separated on 16% SDS-PAGE and analyzed by Western blot analysis.

¹²⁵I-HB-EGF binding assays. ¹²⁵I-HB-EGF was prepared using a modification of the Bolton-Hunter method (33). Labeled HB-EGF was equally active as unlabeled HB-EGF in an assay measuring its stimulation of DNA synthesis in BALB/c 3T3 cells. Equilibrium binding of ¹²⁵I-HB-EGF was conducted on pulmonary fibroblast cultures treated with elastase for different times and at different concentrations. Immediately after elastase treatment, DMEM containing 5% FBS was added back to the cells to inhibit any residual elastolytic activity. The medium was replaced once again before the start of subsequent binding assay. Cultures were incubated for three times with ice-cold DMEM containing 25 mM HEPES and 0.05% gelatin and were incubated on ice for 10 min. ¹²⁵I-HB-EGF was added to each cell well at a final concentration of 5 ng/ml, and the cells were incubated on ice at 4°C for 2 h. HSPG- and receptor-bound ¹²⁵I-HB-EGF were determined separately using a combination of high-salt and...
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low-pH extraction buffers (13). HSPG-bound 125I-HB-EGF was recovered by briefly washing the cells with buffer containing 20 mM HEPES (pH 7.4) and 2 M sodium chloride, followed by a second wash with PBS. Receptor-bound 125I-HB-EGF was recovered by washing the cells twice with buffer containing 25 mM sodium acetate (pH 4.0) and 2 M sodium chloride. Nonspecific binding was determined for each extraction method with control cells by competing with increasing concentrations of unlabeled HB-EGF until no further reduction in label binding was observed (100 μg/ml). Less than 1% of the added 125I-HB-EGF was bound nonspecifically within the HSPG and receptor fractions, and these values were subtracted from all data.

Isolation and analysis of RNA. Total RNA from cells cultured in 75-cm² flasks was isolated with 4 M guanidinium thiocyanate as previously described (52). A single-step procedure using TRIzol reagent (GIBCO Life Sciences, Gaithersburg, MD) was used to isolate total RNA from cells cultured in 35-mm dishes. Equal amounts (10 μg unless otherwise specified) of RNA were fractionated in a 1% agarose–1.8% formaldehyde gel and transferred onto a nylon membrane (Schleicher and Schuell, Keene, NH) by capillary force. RNA was cross-linked to the membrane by ultraviolet irradiation. The membrane was hybridized with 32P-labeled cDNAs. Rat cDNA encoding tropoelastin was described earlier (43). Rat cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from American Type Culture Collection (Manassas, VA). Mouse histone 3/2b plasmid (data not shown), suggesting that the two higher-molecular-weight bands represent modified HB-EGF. The identities of the other two minor bands (14.5, 24, and 25 kDa) are unclear. However, very little, if any, immunoreactive signal was present within the sodium bicarbonate buffer collected from mock-treated cultures. Interestingly, recombinant human HB-EGF purified from s/21 insect cells (R&D Systems) was recognized as three major species (14.5, 18, and 21 kDa). Heterogeneity of HB-EGF has been reported by others and is attributed to different NH₂-terminal truncations (9, 20) and/or different levels of glycosylation (16). The major band (~18–19 kDa) within the elastase digest migrated with similar electrophoretic mobility to the 18-kDa form of the recombinant human HB-EGF. The identities of the other two minor bands (~24 and 25 kDa) are unclear. However, all immunoreactive bands disappeared after the antibody was incubated first with the competing peptide (data not shown), suggesting that the two higher-molecular-weight bands represent modified HB-EGF forms. As expected, we also observed an elastase-dependent release of FGF-2 (Fig. 1B). Overall, these results provide evidence that HB-EGF as well as FGF-2 are released from pulmonary fibroblast cultures by elastase.

Preparation of nuclear extracts and cell lysates. Nuclear extracts were prepared as described (7). Briefly, cell layers were rinsed twice with prechilled Puck’s saline and scraped with cold lysis buffer (containing 10 mM Tris·HCl, pH 7.6, 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40). The cell suspension was centrifuged at 560 g for 10 min, and the supernatant was discarded. The cell pellet was resuspended in the lysis buffer, and the suspension was centrifuged at 560 g for 10 min. After removing the supernatant, the remaining pellet (crude nuclei) was resuspended with the nuclear protein extraction buffer [20 mM HEPES, pH 7.9, 0.35 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, pH 8.0, 25% glycerol, 0.5 mM dithiothreitol, 0.5 μg/ml (1 μM) leupeptin, 2.0 μg/ml (0.3 μM) aprtein, 0.7 μM/ml (1 μM) pepstatin, 0.2 mM sodium vanadate, 100 μM sodium fluoride, 1 μM DFP] at 0.5–1 volume of the pellet size. The extract was incubated on ice for at least 15 min and then centrifuged for 20 min at 9,500 g. The supernatant was transferred into another tube and stored at ~80°C. Total cell lysates were prepared with ice-cold lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, pH 7.5, 1 mM EGTA, pH 9.0, 0.5% Nonidet P-40, 0.4 mM PMSF, 0.2 mM sodium vanadate) as described (6). Protein concentrations were determined by the biocinonic acid assay (Pierce, Rockford, IL).

Western blot analysis. Unless otherwise specified, 40 μg of nuclear extracts or cell lysates were separated on 10% SDS-PAGE before being electrotransferred overnight to nitrocellulose membranes (Schleicher and Schuell). The membranes were stained in Ponceau S dye (Sigma, St. Louis, MO) briefly to examine equal protein loading and transfer. The membranes were then blocked with 5% milk in TBST (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) before being probed with the primary antibody at room temperature for 2 h or at 4°C overnight. After being washed with TBST, the membranes were incubated with the secondary antibody for 45 min. Specific proteins were visualized by the chemiluminescence method according to the manufacturer’s instructions (Kirkgegaard & Perry Laboratories, Gaithersburg, MD). To probe the blot with a second antibody, the membrane was incubated in 68°C stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris·HCl, pH 6.8) for 30 min to remove any residual primary and secondary antibodies.

RESULTS

HB-EGF is released by elastase digestion of pulmonary fibroblasts. We first analyzed whether HB-EGF is present among the products released by elastase digestion. Two-week-old, matrix-laden pulmonary fibroblasts were treated with 5 μg/ml of pancreatic elastase for 5 min. Elastase-released material was collected and subjected to Western blot analysis using an HB-EGF-specific antibody (Fig. 1A). Several immunoreactive bands were detected within the elastase digest. Very little, if any, immunoreactive signal was present within the sodium bicarbonate buffer collected from mock-treated cultures. Interestingly, recombinant human HB-EGF purified from s/21 insect cells (R&D Systems) was recognized as three major species (14.5, 18, and 21 kDa). Heterogeneity of HB-EGF has been reported by others and is attributed to different NH₂-terminal truncations (9, 20) and/or different levels of glycosylation (16). The major band (~18–19 kDa) within the elastase digest migrated with similar electrophoretic mobility to the 18-kDa form of the recombinant human HB-EGF. The identities of the other two minor bands (~24 and 25 kDa) are unclear. However, all immunoreactive bands disappeared after the antibody was incubated first with the competing peptide (data not shown), suggesting that the two higher-molecular-weight bands represent modified HB-EGF forms. As expected, we also observed an elastase-dependent release of FGF-2 (Fig. 1B). Overall, these results provide evidence that HB-EGF as well as FGF-2 are released from pulmonary fibroblast cultures by elastase.

Elastase-treatment decreases the binding of HB-EGF at cell surface receptors. We next examined whether elastase treatment affected HB-EGF binding to pulmonary fibroblasts. Because HB-EGF binds to both HSPG and receptor sites, we investigated the time- and dose-dependent effects of elastase treatment on HB-EGF binding at both sites (Fig. 2). Elastase addition resulted in a time-dependent decrease in HB-EGF binding at both HSPG (Fig. 2A) and receptor sites (Fig. 2B).
released HB-EGF is likely to have only a minimal effect on the injured cells immediately after enzyme treatment.

**HB-EGF downregulates elastin mRNA via EGFR activation.** To determine how HB-EGF might individually affect elastin expression in pulmonary fibroblasts, we used matrix-laden cultures that were not treated with elastase in experiments similar to those in which we have studied FGF-2 effects (44). Cells were treated with HB-EGF for various times (Fig. 3A) or with different concentrations (Fig. 3B). Total RNA was isolated from control and treated cultures, and Northern blot analyses were performed to measure elastin mRNA levels in these cultures. HB-EGF downregulated elastin mRNA in a time- and dose-dependent manner. As shown in Fig. 3A, elastin mRNA down-regulation became visible 8 h after ligand treatment, and the maximal inhibitory effect was seen at 48 h. As a control, GAPDH and histone 3/2b mRNA levels were not affected within the 48-h period. The unresponsiveness of an S-phase marker such as histone 3/2b mRNA to HB-EGF treatment is consistent with our previous observations that the primary pulmonary fibroblasts are “contact inhibited” (42). The effect of HB-EGF on elastin was saturated at a concentration of 10 ng/ml (Fig. 3B).

We then examined whether EGFR activation is involved in HB-EGF-induced downregulation of elastin mRNA by treating cells with AG-1478, a selective tyrosinе-tyrosine kinase inhibitor (35). AG-1478 treatment abolished elastin mRNA downregulation induced by HB-EGF, but not by FGF-2, which signals via a different receptor tyrosine kinase (Fig. 3C). These results indicate that activation of EGFR tyrosine kinase is an integral part of the HB-EGF-induced signaling pathway leading to downregulation of elastin mRNA.

**HB-EGF inhibits elastin transcription.** We next examined whether HB-EGF-induced downregulation of elastin mRNA is caused by transcriptional suppression. Elastin gene transcription levels in control and HB-EGF-treated cells were measured by nuclear run-on analyses. As shown in Fig. 4A, HB-EGF treatment led to a significant decrease (74.2 ± 6.4%) in the level of elastin transcription in 48 h, whereas the transcription level of actin was not affected. Quantitation of the data shows that after 48 h of HB-EGF treatment, elastin gene transcription was inhibited to an extent comparable with the decrease of elastin mRNA (Fig. 4B). A time course of elastin transcription levels in response to HB-EGF is given in Fig. 4C. The effect on elastin transcription was apparent as early as 4 h (∼20% inhibition), suggesting that this is a primary effect of HB-EGF and not a secondary downstream event. We conclude from these data that transcriptional inhibition accounts for HB-EGF-dependent downregulation of elastin mRNA.

**HB-EGF induces ERK1/2 activation and subsequent Fra-1 nuclear accumulation.** EGFR activation can induce a number of signal transduction pathways (39). Among these, the best-characterized pathway is the
ERK1/2 pathway. In this pathway, EGFR tyrosine phosphorylation initiates a signaling cascade that involves sequential activation of Ras, c-Raf, MEK1/2, and ERK1/2, which in turn transduces the signal into the nucleus. We first examined whether ERK1/2 is activated by HB-EGF signaling in these cells. Cell lysates were prepared after different periods of HB-EGF treatment and subjected to Western blot analysis. Figure 5A shows that HB-EGF induced a time-dependent phosphorylation of ERK1/2, with the levels of phosphorylated ERK1/2 (pERK1/2) peaking at 15–30 min and returning close to basal levels at 10 h. Levels of total ERK1/2 did not change in response to HB-EGF treatment.

Activator protein-1 transcription factors are among the primary targets of ERK1/2 activation (51). We have previously shown, in the same cells, that FGF-2-induced ERK1/2 signaling leads to phosphorylation of Elk-1 and induction of Fra-1, which results in increased c-Jun/Fra-1 heterodimer complex binding to a distal negative element in the elastin promoter (6, 42). We examined whether similar nuclear signaling is induced by HB-EGF. Cells were treated with HB-EGF for different times, and the levels of nuclear pERK1/2 and Fra-1 were evaluated by Western blot analysis (Fig. 5B). HB-EGF treatment resulted in a time-dependent increase in nuclear pERK1/2 levels, whereas no apparent changes in the levels of total ERK1/2 were observed. The level of Fra-1 was significantly elevated after 6 h of HB-EGF treatment, and it was still clearly above basal level at 24 h. The levels of c-Jun, a binding partner of Fra-1, were not affected. Figure 5C presents the Ponceau S staining of two representative lanes.

Fig. 2. Elastase decreases HB-EGF binding to both heparan sulfate proteoglycan (HSPG) and receptor sites. Pulmonary fibroblasts were treated with 0.5 μg/ml of porcine pancreatic elastase for increasing times (A and B) or with increasing elastase concentrations (C and D) for 15 min at 37°C. The cells were incubated with 5 ng/ml of 125I-HB-EGF for 2 h at 4°C on ice. The label was removed, and the cells were washed 3 times with ice-cold binding buffer. To isolate the HSPG-bound fraction (A and C), the cells were washed once with 20 mM HEPES (pH 7.4) containing 2 M sodium chloride and once with PBS. To isolate the receptor-bound fraction (B, C and D), the cell layers were washed 2 additional times with 25 mM sodium acetate (pH 4.0) containing 2 M sodium chloride. Data are expressed as means ± SE from quadruplicate samples. Nonspecific binding was determined for each extraction method with control cells by competing with increasing concentrations on unlabeled HB-EGF until no further reduction in label binding was observed (100 μg/ml), and nonspecific bound values were subtracted from all data presented.

Fig. 3. Effect of HB-EGF on elastin mRNA levels. Pulmonary fibroblast cultures were treated with 10 ng/ml of HB-EGF for the indicated times (A) or with various concentrations of HB-EGF for 48 h (B) and total cellular RNA was prepared. RNA from each sample (10 μg) was analyzed by Northern blot analysis with 32P-labeled cDNA probes for elastin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or histone 3/2b. C: pulmonary fibroblasts were pretreated with 10 μM AG-1478 for 1 h. HB-EGF (10 ng/ml) or FGF-2 (10 ng/ml) was added, and the cells were harvested for total RNA isolation 24 h later. Elastin mRNA levels were determined by Northern blot analysis. Results shown are representative of 3 independent experiments. C, control.
loaded with either nuclear extract or total cell lysate, indicating the uniqueness of protein distribution in the nuclear compartments.

MEK1/2 inhibitor PD-98059 (12) was used to test the functional linkage between ERK1/2 activation and Fra-1 induction. PD-98059 inhibited HB-EGF-induced ERK1/2 activation in a dose-dependent manner (Fig. 5D). We then examined the effect of PD-98059 on HB-EGF-dependent Fra-1 induction. As shown in Fig. 5E, the majority of HB-EGF-initiated Fra-1 induction

Fig. 4. Transcriptional inhibition of elastin by HB-EGF. A: pulmonary fibroblast cultures were treated with 10 ng/ml of HB-EGF for 48 h. Nuclei from control and treated cultures were isolated. Nuclear run-on reaction was performed in the presence of [α-32P]UTP for 20 min. Equal counts of newly synthesized transcripts were used to probe the nitrocellulose filters on which actin, elastin cDNAs, and pBluescript plasmid were immobilized. The filters were then exposed to X-ray film at −80°C for 1 wk. B: quantitation of elastin mRNA and transcription levels in cells treated with or without HB-EGF for 48 h. Quantitation of data from 5 independent Northern analyses and 3 independent nuclear run-on assays is presented. Levels of elastin mRNA and transcription in HB-EGF-treated cells are shown as relative to those in control cells. Data are given as means ± SD. C: elastin transcription levels in cells treated with HB-EGF for different times were measured by nuclear run-on analyses.

Fig. 5. HB-EGF activates ERK1/2 and induces Fra-1. A: pulmonary fibroblast cultures were treated with 10 ng/ml of HB-EGF for the indicated times. Cell lysates were analyzed by Western blot analysis with antibodies against phosphorylated ERK1/2 (pERK1/2) or ERK1/2. B: cells were treated with 10 ng/ml of HB-EGF for the indicated times, and nuclear extracts were prepared. Nuclear proteins (40 μg) were subjected to Western blot analysis with antibodies specific for pERK1/2, ERK1/2, Fra-1, and c-Jun. C: Ponceau S staining of representative lanes loaded with equal amounts of nuclear extracts (NE) or cell lysates (CL). D: pulmonary fibroblasts were preincubated for 1 h with or without various concentrations of PD-98059 as indicated. Cells were then treated with or without 10 ng/ml of HB-EGF. Total cell lysates were prepared 30 min after HB-EGF addition, and the levels of pERK1/2 were shown by Western analysis. E: pulmonary fibroblasts were treated with 10 ng/ml of HB-EGF for 24 h in the presence or absence of 50 μM PD-98059. Nuclear extracts were prepared, and 40 μg of nuclear proteins from each sample were subjected to Western blot analysis with antibody specific for Fra-1. Results are representative of 3 independent experiments.
was blocked by PD-98059, indicating that the nuclear accumulation of Fra-1 is downstream of ERK1/2 activation. The residual Fra-1 induction is likely due to PD-98059’s incomplete inhibition of ERK1/2 activation, as evidenced in Fig. 5D.

Because PD-98059 did not completely inhibit HB-EGF-induced ERK1/2 activation, we tested the effect of another MEK1/2 inhibitor, U-0126 (14). U-0126 inhibited HB-EGF-induced ERK1/2 activation more efficiently than PD-98059 (Fig. 6A vs. Fig. 5D), which is consistent with other reports (14, 34). Total ERK1/2 levels were not altered by U-0126 treatment. Cells were then preincubated with 25 μM U-0126 and treated for 24 h with HB-EGF, and elastin mRNA levels in these cells were measured. As shown in Fig. 6B, U-0126 abrogated HB-EGF-dependent elastin downregulation, indicating that the ERK1/2 pathway mediates HB-EGF-induced downregulation of elastin gene expression.

**HB-EGF and FGF-2 have additive inhibitory effects on elastin gene expression.** Because FGF-2 and HB-EGF are released by elastase, and both are potent elastin repressors, we examined the effect of the addition of the two ligands together on elastin mRNA levels. Each ligand was used at a concentration of 10 ng/ml, which represented their maximal inhibitory effects on elastin mRNA (Fig. 3B and data not shown). Cells were treated for 48 h with HB-EGF, FGF-2, or the two ligands in combination. Interestingly, coaddition of these two ligands resulted in a greater inhibitory effect on elastin mRNA than observed with either ligand alone (Fig. 7). GAPDH levels were not affected by any of the treatments. These results show that the elastin-suppressing effects of these two elastase-released growth factors are additive.

**HB-EGF induces FGF-2 mRNA and protein levels.** HB-EGF is thought to indirectly regulate some cellular processes by inducing the levels of other growth factors (2, 36, 53). Because of the major contribution of FGF-2 as an elastin downregulator in pulmonary fibroblasts (44), we examined whether HB-EGF treatment induces FGF-2, which may potentially contribute to HB-EGF-dependent inhibition of elastin transcription. Cells were treated with HB-EGF for different times. Total cellular RNA was prepared and subjected to Northern analysis to measure the levels of FGF-2 mRNA. As shown in Fig. 8A, HB-EGF treatment led to a time-dependent induction of FGF-2 mRNA, as the level of FGF-2 mRNA was increased gradually to its peak in 8–12 h. Next, cellular FGF-2 protein levels in HB-EGF-treated cells were examined by Western analysis. In pulmonary fibroblast cell lysates, three forms of FGF-2 were detected, with apparent molecular masses of 18, 22, and 23.5 kDa (Fig. 8B), very similar to those described by others as different products of translation initiated from alternative start codons (37). HB-EGF treatment increased the levels of all three forms of FGF-2 protein. The induction was visible at

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**Fig. 6.** HB-EGF downregulates elastin gene expression in an ERK-dependent manner. Cells were pretreated for 1 h with various concentrations of U-0126 as indicated and then treated with 10 ng/ml of HB-EGF for 30 min (A) or 24 h (B). A: total cell lysates were prepared and subjected to Western blot analysis using antibodies against pERK1/2 or ERK1/2. B: total RNA was prepared and analyzed by Northern blot analysis for elastin mRNA levels. Two independent experiments were performed, and the representative results are shown.

**Fig. 7.** Additive effect of HB-EGF and FGF-2 on elastin gene downregulation. Cells were treated with HB-EGF (10 ng/ml), FGF-2 (10 ng/ml), or the two ligands in combination (H+F; 10 ng/ml for each ligand) for 48 h, and total cellular RNA was isolated. RNA from each sample (10 μg) was analyzed by Northern blot analysis for elastin and GAPDH levels. Representative results from 3 independent experiments are shown.

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Fig. 8. Induction of FGF-2 mRNA and protein levels by HB-EGF. Cells were treated with HB-EGF (10 ng/ml) for the indicated times. Total cellular RNA and cell lysates were prepared. A: equal amount (30 μg) of total cellular RNA was analyzed on a Northern blot for FGF-2 mRNA levels. Methylene blue staining of the membrane is provided to show equal RNA loading. B: aliquots of cell lysates (100 μg) were separated on a 16% SDS-PAGE and analyzed on a Western blot for FGF-2 protein levels. Human recombinant FGF-2 (SciosNova, 18-kDa form) was loaded on the far left lane as a positive control, and an arrowhead indicates its position. The 3 different forms of cytoplasmic FGF-2 are indicated (*). Positions of several molecular weight markers are shown by arrows. Ponceau S (Sigma) staining of the membrane is provided to show equal protein loading. Results are shown as representative of 3 independent experiments.

8 h and maximal at 24 h. Together, these results demonstrate the potential of the two elastase-released growth factors to work together in regulating elastin gene expression.

DISCUSSION

Uninhibited activity of proteases and subsequent loss of elastic fibers within alveolar walls are major contributing factors in the pathogenesis of COPD. Insufficient or compromised repair of degraded elastin and interstitial cell apoptosis are thought to exacerbate disease progression (21, 25, 26). Previously, we have examined the elastogenic response of cultured pulmonary fibroblasts to brief elastase exposure (41) or to the addition of elastase-released products (15), with each situation mimicking cellular response at the site of injury or distal from the injury, respectively. Subsequently, we identified FGF-2 as a major growth factor released by elastase from pulmonary fibroblast cultures and characterized the mechanism of FGF-2-induced elastin transcriptional repression (6, 42, 44). In the present study, we have pursued characterization of another growth factor released by elastase, i.e., HB-EGF.

Addition of HB-EGF to undigested pulmonary fibroblasts resulted in a significant decrease of elastin gene transcription through ERK activation. Furthermore, HB-EGF treatment led to a time-dependent induction of Fra-1, although not affecting the levels of its binding partner, c-Jun. These signaling events are similar to those described for FGF-2 (6, 42), implying that a common nuclear signaling pathway is used by both ligands to inhibit elastin transcription. These results suggest that elastase-released HB-EGF, together with FGF-2 (44), may act in a paracrine manner to repress elastin gene expression.

Like other EGF family members, soluble HB-EGF is processed from its transmembrane precursor, i.e., pro-HB-EGF (40). Interestingly, pro-HB-EGF by itself is biologically active and can function as a juxtacrine factor (19). The membrane-anchored proform signals only in adjacent cells, whereas its cleaved product can function in a paracrine mode and impact nonadjacent cells. Besides the obvious physical difference between their modes of action, it has been demonstrated that the biological effects of the membrane-bound form vs. the soluble, released form of HB-EGF can be different (24). Together, these observations led to the hypothesis that shedding or proteolytic release of soluble HB-EGF from the cell surface can act as a switch to regulate certain biological processes. Several metalloproteinases from the ADAM (a disintegrin and a metalloproteinase) family are thought to be responsible for HB-EGF ectodomain shedding in different cell types (17). By using a hydroxamate-based inhibitor of EGFR ligand shedding and the neutralizing antibody against HB-EGF, others have suggested the involvement of HB-EGF shedding in submandibular gland epithelial morphogenesis (49) and cutaneous wound healing (47). In the latter study, which used a model based on mechanical disruption of the cell culture layer, HB-EGF processing was found to be associated with keratinocyte proliferation and migration during the repair process (47).

In the present study, we show that brief treatment of matrix-laden pulmonary fibroblasts with elastase results in the release of soluble HB-EGF that is similar in size to the shed form of HB-EGF reported by others (16). This implies that pancreatic elastase may cleave a proteolytic-susceptible site in pro-HB-EGF similar to that targeted by the HB-EGF “sheddase” or activate a metalloproteinase, which in turn processes pro-HB-EGF. Interestingly, others have shown that elastases release the mature form of TGF-α, another EGF family member, from the cell surface (28, 30). These observations, together with our findings, suggest that elastase treatment of cells can lead to processing of membrane-bound growth factor precursors, with the end products similar to those resulting from metalloprotease-mediated ectodomain shedding.

Elastase-released HB-EGF may function to induce cell proliferation and migration at the matrix-injured...
sites, similar to that reported by Tokumaru and colleagues (47) for an in vitro wound model. It is important to note that the equilibrium binding of HB-EGF to elastase-treated cells was decreased immediately after the injury due to the loss of cell-associated HSPG (Fig. 2). Studies of FGF-2 binding to elastase-treated cells show that the number of HSPG sites on the cell surface gradually recovers after the initial loss (4). Therefore, elastase-treated cells can be responsive to the signal of HB-EGF if given time for recovery. Previous studies from our group have proposed that the cellular responses, such as those induced by growth factors, are dependent on the proliferative potential of the cells (6). In this regard, it is significant to point out that we have recently reported that one of the major effects of elastase treatment is to release cells from contact inhibition, therefore reinitiating both cell proliferation and subsequent communication between the cells and the newly synthesized matrix components (41). Thus although in undigested matrix-laden fibroblasts the major effect of HB-EGF is to inhibit elastin gene expression, different cellular responses, such as proliferation or increased migration, can be induced in cells primed with brief elastase treatment. Whether soluble HB-EGF plays a role in inducing cell proliferation and/or recruitment of cells to the site of injury is a question we are investigating.

Because FGF-2 and HB-EGF are both released by elastase, we investigated the effect of coaddition of the two growth factors on elastin gene expression. We found that FGF-2 and HB-EGF have additive inhibitory effects on elastin gene expression. This is an interesting observation considering the similarity between the two signaling pathways. It suggests that pulmonary fibroblasts have a sufficient intracellular signaling capacity to quantitatively respond to simultaneous activation of EGFR and FGFR receptor. Furthermore, we demonstrate that HB-EGF induces the synthesis of FGF-2 in pulmonary fibroblast cultures, suggesting that the two factors participate in cross-regulatory mechanisms to mediate cellular responses in the pulmonary fibroblasts. Interestingly, cross-regulation between FGF-2 and HB-EGF has been shown in vascular smooth muscle cells (11, 36) and lung fibroblasts (53). The latter study shows that the induced FGF-2 was involved in the proliferation of lung fibroblasts stimulated by HB-EGF. How FGF-2 and HB-EGF might function together to promote repair of elastase injury is an intriguing question and one we are pursuing.

In conclusion, our results demonstrate that HB-EGF is a potent regulator of elastin gene expression in “normal” undigested fibroblast cell cultures. The fact that HB-EGF downregulates elastin gene expression by a mechanism that appears similar to FGF-2, albeit via activating a different receptor, is interesting. A more complicated situation that we are currently addressing is how these released growth factors impact cells within areas that have sustained elastolytic injury.

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**DISCLOSURES**

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**REFERENCES**


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