Elastase-mediated Release of Heparan Sulfate Proteoglycans from Pulmonary Fibroblast Cultures

A MECHANISM FOR BASIC FIBROBLAST GROWTH FACTOR (bFGF) RELEASE AND ATTENUATION OF bFGF BINDING FOLLOWING ELASTASE-INDUCED INJURY*

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We have investigated elastase-mediated alterations in the expression of basic fibroblast growth factor (bFGF) receptors and proteoglycan co-receptors and characterized the subsequent effects on bFGF receptor binding profiles. For these studies, pulmonary fibroblast cultures were treated with porcine pancreatic elastase, and elastase-mediated changes in bFGF receptor expression and binding profiles were assessed. Quantitation of [35S]sulfate-labeled proteoglycan and total glycosaminoglycan release from fibroblast matrices indicated that elastase treatment released sulfated proteoglycan from the cell surface in a time- and dose-dependent fashion that correlated strongly with elastase-mediated bFGF release. Ligand binding studies indicated that elastase treatment decreased total binding of 125I-bFGF to the cell surface and affected both fibroblast growth factor receptor and heparan sulfate proteoglycan (HSPG) binding sites. Western blot analyses indicated that elastase treatment did not release significant amounts of fibroblast growth factor receptor protein. These findings indicate that elastase-mediated HSPG release from fibroblast matrices reduces the effective affinity of bFGF for its receptor. Collectively, these studies suggest that HSPG co-receptors are important mediators of the pulmonary fibroblast response to elastase treatment and that bFGF, HSPG, and other elastase-released entities play an important role in the response of the lung to chronic injury.

Elastin is an essential extracellular matrix protein that confers important structural and functional features to the lung. During early stages of development, pulmonary elastin is synthesized as a protein precursor, tropoelastin, which is secreted from the cell and undergoes covalent cross-linking by lysyl oxidase to form insoluble elastin fibers within the extracellular matrix (1–3). Under normal conditions, once the protein has been synthesized and deposited within the matrix, there is very little turnover of elastin within the adult lung (4). With the onset of pulmonary obstructive diseases such as emphysema, there is a progressive loss of elastic fibers from the alveolar wall (5, 6). This loss of elastin is believed to result from chronic injury to the lung due to the release of several proteolytic enzymes, including elastases, from infiltrating inflammatory cells recruited to the site of damage. Whereas damaged elastin fibers can be repaired under acute elastase exposure conditions (7), this repair process is believed to be insufficient under conditions of chronic injury leading to disease.

Several animal models have provided valuable insight into the clinical, biochemical, and pathological manifestations of pulmonary emphysema (8). However, due to the presence of multiple cell types present in the lung, attempts to investigate specific molecular and cellular aspects of the disease process in vivo have been limited by the complexity of the system. The use of pulmonary fibroblast cell cultures has provided a unique model system for investigating specific cellular responses to protease-induced injury (9, 10). Basic fibroblast growth factor (bFGF), one factor released from the extracellular matrix in response to elastase exposure, has been demonstrated to have potent, negative regulatory effects on elastin steady-state mRNA and protein levels in pulmonary fibroblast cultures and has been identified as a central mediator of elastin repair (11, 12). bFGF, a member of the heparin binding growth factor family, elicits its biological response by binding to cell surface receptors, which leads to receptor activation and initiation of cell signaling events (13). In addition to binding to its cell surface receptors, bFGF also binds to heparan sulfate proteoglycan (HSPG) co-receptors that facilitate bFGF-receptor interactions and protect bFGF from proteolytic degradation within the extracellular environment (14–17). Whereas a role for bFGF under conditions of elastase-mediated injury has been established, the mechanism for bFGF release and the consequent effects of elastase on FGF receptor expression and function are not well understood.

The focus of this study was to investigate changes in the expression of cell surface bFGF receptors, HSPG co-receptors, and bFGF cellular binding profiles in pulmonary fibroblast cultures after elastase treatment. The results from this study indicate that treatment of pulmonary fibroblasts with elastase selectively releases cell surface heparan sulfate proteoglycans, which results in a net decrease of bFGF receptor binding.

**EXPERIMENTAL PROCEDURES**

Neonatal Rat Lung Pulmonary Fibroblast Cell Cultures—Primary cultures of pulmonary fibroblasts were isolated from the lungs of 3-day-old Harlan Sprague-Dawley rats using established protocols (9, 10), and the cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 1,000 units/ml penicillin, and 100 µg/ml streptomycin. For all studies, the cells were maintained for 10–12 days after the second

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† The abbreviations used are: bFGF, basic fibroblast growth factor; GAG, glycosaminoglycan; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; FGF, fibroblast growth factor; PAGE, polyacrylamide gel electrophoresis.
passage before the start of all experiments.

**Elastase Treatment of Fibroblast Cultures**—At the onset of the experiment, cell cultures were rinsed once with phosphate-buffered saline and twice with 44 mM sodium bicarbonate buffer, pH 7.4. Cells were treated with 44 mM sodium bicarbonate buffer supplemented with or without porcine pancreatic elastase for 15 min at 37 °C as indicated. The elastase concentrations used were based on previous studies that established the range of elastase needed to induce proteolytic damage to the elastin matrix without causing cytotoxic effects (10, 12). Similar results were obtained using human neutrophil elastase preparations (data not shown). The elastase and bicarbonate solutions were removed from cell layers, and diisopropyl fluorophosphate was added to a final concentration of 1 μM to inhibit residual elastase activity in the digested fractions. Dulbecco's modified Eagle's medium containing 1% fetal bovine serum was added to the cell cultures to inhibit residual elastolytic activity. The cells were incubated on ice for 10 min before the start of subsequent analyses or frozen in liquid nitrogen before extraction.

**Extraction of Elastase-treated Cells**—Control and elastase-treated cells were extracted by scraping with a cell lifter in 150 mM sodium chloride, 50 mM Tris, pH 8.0, 1 mM sodium orthovanadate, 1% Triton X-100, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, and 10 μg/ml leupeptin. Cell lysates were centrifuged at 12,000 × g, and the supernatants were collected and assayed for protein content using the Bio-Rad protein assay to standardize the protein amounts applied to the gel.

**bFGF Immunoassay**—bFGF concentrations in elastase-digested fractions were determined using a commercially available kit from R&D Systems (Minneapolis, MN).

**Quantitation of Sulfated Glycosaminoglycan Content**—Total sulfated glycosaminoglycan (GAG) content in elastase-digested fractions was determined by spectrophotometric analysis using the dimethylmethylene blue assay (18). Unknown GAG concentrations were determined from standard curves of chondroitin sulfate.

**35S Metabolic Labeling and Quantitation of Cellular Proteoglycans**—50 μCi/ml [35S]sulfate was added to pulmonary fibroblast cultures 24 h before elastase treatment. [35S]Labeled cells were treated with elastase and extracted with 10 mM Tris, pH 8.0, 1 mM urea, 0.1% Triton X-100 and 1 mM sodium fluoride. 35S-labeled heparan sulfate proteoglycans were quantitated by vacuum filtration through cationic nylon (ZetaProbe; Bio-Rad) and nitrous acid treatment (19).

**Heparinase III Treatment of Elastase-digested Fractions**—Heparinase III was generously provided by Dr. Elizabeth Denholm (Ibex Technologies, Inc, Montreal, Canada). 250 μl aliquots of elastase-digested fractions were incubated for 3 h at 37 °C with 0.1 unit/ml heparinase III (EC 4.2.2.8) in 100 mM sodium phosphate buffer, pH 7.0, containing 150 mM sodium chloride. Control digests were incubated without the enzyme under the same conditions. At the end of the incubation period, samples were precipitated in ice-cold acetone overnight at −20 °C. The precipitated fractions were dried and resuspended in 100 μl of Laemmli sample buffer and boiled for 5 min at 100 °C before SDS-PAGE and Western blot analysis.

**SDS-PAGE and Western Blot Analysis**—Elastase-digested fractions and total cell lysates were subjected to SDS-PAGE (20) before electrotransfer onto Millipore Immobilon membranes (Milford, MA) (21). For Western blot analysis, the membranes were blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20. Primary antibody incubations were performed at room temperature for 1 h. The murine monoclonal antibodies used in the present studies were as follows: anti-heparan sulfate (clone 3G10), anti-FGF receptor 1 (clone 19B2), and anti-syndecan-4 (clone 150.1; which was generously provided by Dr. John Couchman, University of Birmingham, Birmingham, AL). The blots were developed using chemiluminescent detection methods (ECL; Amersham Pharmacia Biotech).

**[125I]-bFGF Binding Studies**—[125I]-bFGF was prepared using a modification of the Bolton-Hunter method (22), and equilibrium binding of [125I]-bFGF was conducted on pulmonary fibroblast cell cultures immediately after elastase treatment. Elastase-treated cell cultures were rinsed three times with ice-cold binding buffer (Dulbecco's modified Eagle's medium, 25 mM HEPES, and 0.05% gelatin) and incubated on ice at 10 μM [125I]-bFGF was added at each concentration to a total of 1 N NaOH. All coverslips were treated with fluorescein-conjugated goat anti-mouse IgG secondary antibody at a final dilution of 10 μg/ml in 1% bovine serum albumin-phosphatebuffered saline for 1 h at 37 °C. Fluorescence was examined using an Olympus IX70 fluorescence microscope. Cell cultures stained with secondary antibody alone were used to establish the fluorescence exposure settings, and these settings were used to analyze the cell cultures stained for heparan sulfate.

**RESULTS**

**Elastase Mediates the Release of HSPGs from Pulmonary Fibroblast Matrices**—We have previously reported that elastase treatment of fibroblast cultures results in the release of active bFGF from the extracellular matrix (12). Because bFGF is known to be sequestered in the extracellular matrix through binding to heparan sulfate proteoglycans, elastase might be able to release bFGF by digesting proteoglycan core proteins. Therefore, the ability of elastase to release GAG from pulmonary fibroblasts was analyzed and compared with the release of bFGF. Elastase treatment resulted in a dramatic release of GAG over a range of concentrations where bFGF release was observed (Fig. 1). Exposure of pulmonary fibroblast cell cultures to 0.5 μg/ml elastase for 15 min stimulated the release of GAG by 6-fold compared with that released in the absence of elastase. The addition of 10 μg/ml elastase led to a maximal 10-fold increase in the release of GAG compared with control cultures (Fig. 1A). The amounts of bFGF were also measured in these same samples using a quantitative enzyme-linked immunosorbent assay (Fig. 1B). Elastase stimulated bFGF release dramatically over this concentration range, with maximal release being observed at 10 μg/ml (~15-fold increase compared with control cultures). The release of GAG from fibroblast cultures by elastase treatment correlated directly with elastase-mediated bFGF release (r2 = 0.956; Fig. 1C). Thus, the mechanism of elastase-mediated bFGF release is likely through disruption of matrix proteoglycans.

The ability of elastase to induce the release of GAG was characterized in detail using several approaches. Pulmonary fibroblast cultures were radiolabeled metabolically with [35S]sulfate for 24 h before elastase treatment so that the release of newly synthesized GAG from the cell/extracellular matrix could be quantitated simultaneously with the measurement of GAG appearance in the soluble form. The elastase treatment solutions and cell/extracellular matrix layers were extracted and analyzed for [35S]sulfate-labeled proteoglycan content by filtration through cationic nylon membranes. Elastase caused a dose-dependent loss of cell/extracellular matrix associated [35S]-GAG and a concomitant increase in soluble [35S]-GAG (Fig. 2). The fraction of the total [35S]-GAG that constituted heparan sulfate (35–55%) in the cell/extracellular matrix extracts and the elastase treatment solutions did not vary with elastase treatment (data not shown).

It is possible that elastase releases sulfated GAG by proteolytically digesting the protein cores of the cell-associated proteoglycans. To determine whether elastase treatment caused the release of heparan sulfate proteoglycan core fragments, we conducted immunohisto analyses on elastase extracts. We used an antibody (clone 3G10) that recognizes desaturated uronic acid residues remaining on heparan sulfate proteoglycan core.
proteins after heparinase III digestion (24). Consequently, for these studies, elastase-digested fractions were treated with heparinase III to selectively degrade heparan sulfate and generate the reactive epitope. Elastase exposure produced a concentration-dependent increase in the release of heparan sulfate proteoglycan core proteins from pulmonary fibroblast cell cultures. In the absence of elastase exposure, a minimal amount of heparinase III-sensitive material was released from the cell layer (Fig. 3A, lane 1). However, with the addition of elastase concentrations as low as 0.1 μg/ml, the release of HS chains was readily apparent (Fig. 3A, lanes 2–8). Furthermore, it appeared that elastase cleaved many of the core proteins at multiple sites because higher concentrations of elastase led to a progressive increase in low molecular weight core proteins with a decrease in the high molecular weight species. The immunoreactive bands reflected the presence of the 3G10 antibody epitope because identical analyses of samples treated without heparinase III produced no reactive bands. Furthermore, when heparinase III was analyzed in the absence of sample, no immunoreactive bands were observed (data not shown).

Whereas a number of heparan sulfate proteoglycans have been identified, one major class that has been implicated as modulators of bFGF binding and activity are the syndecans (25). Syndecan-4, in particular, has been detected on fibroblast-like cell types (26). Therefore, heparinase III-treated elastase extracts of pulmonary fibroblast cells were analyzed for the presence of syndecan-4. Immunoblots probed with anti-syndecan-4 antibodies revealed the presence of an immunoreactive band with an apparent M_r 548,000 (Fig. 3B). The anomalously large syndecan-4 core protein is consistent with previous reports of SDS-PAGE analysis of this protein (27). As with total GAG and heparan sulfate proteoglycan core protein, increasing elastase concentrations resulted in progressively more syndecan-4 release into the digest fractions. Immunoblot analysis of heparinase III-treated whole cell lysates from elastase-treated cells showed no significant difference in the relative amount of syndecan-4 protein remaining at the cell surface after elastase exposure (data not shown). These results suggest that elastase exposure leads to limited proteolytic cleavage of syndecan-4
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Core proteins. Heparinase III-treated elastase fractions were also subjected to immunoblot analysis using an antibody directed against the major basement membrane heparan sulfate core protein, perlecan. No perlecan core protein was detected in elastase-treated fractions (data not shown).

To directly visualize the elastase-mediated loss of heparan sulfate from the pulmonary fibroblasts, cells were stained with a monoclonal antibody against native heparan sulfate and analyzed by fluorescence microscopy (Fig. 4). Extracellular staining for heparan sulfate chains was readily apparent in control cells and in cells treated with 0.5 μg/ml elastase. The addition of elastase concentrations in excess of 2.5 μg/ml to fibroblast cultures resulted in a dramatic loss of extracellular staining for HS sites. This general decrease in extracellular staining is consistent with the loss of HSPG (Figs. 1 and 2) from the cell surface after elastase treatment. Collectively, these studies demonstrate that elastase induced the release of heparan sulfate proteoglycans from pulmonary fibroblast matrices.

**Elastase Treatment Decreases bFGF Binding to both HSPG and FGF Receptor Sites**—Because bFGF is known to bind to HSPG, and elastase treatment leads to the release of HSPG from the pulmonary fibroblasts, we investigated how the loss of HSPG sites affected the binding of bFGF to these cells. For these studies, fibroblast cultures were treated with increasing concentrations of elastase, and the cells were subjected to 125I-bFGF equilibrium binding analysis. The administration of increasing concentrations of elastase resulted in a dose-dependent decrease in the amount of total 125I-bFGF bound specifically to the cells (Fig. 5). Treatment of fibroblast cultures with 2 μg/ml elastase decreased the total 125I-bFGF binding by 35%, whereas treatment of fibroblast cultures with 40 μg/ml elastase resulted in a 99% loss of 125I-bFGF binding. Total binding of 125I-bFGF was found to recover to 88% of control levels 24 h after elastase treatment (data not shown). Maximal loss of 125I-bFGF binding sites was also found to be time-dependent, with the maximal loss of total binding occurring after 15 min of elastase exposure (data not shown).

Because bFGF can bind to both HSPG and receptor sites, we assessed the effects of elastase treatment on the bFGF receptor and HSPG co-receptor binding sites independently (Fig. 6). For both HSPG and FGF receptor sites, elastase treatment caused a dose-dependent decrease in the binding of 125I-bFGF. Treatment with 2 μg/ml elastase decreased binding to HSPG sites by 54% (Fig. 6A) and decreased binding to receptor sites by nearly 70% (Fig. 6B). Treatment of fibroblast cultures with 40 μg/ml elastase eliminated more than 98% of the binding interactions at both the HSPG and receptor sites. Collectively, these data indicated that elastase treatment of pulmonary fibroblast cultures significantly decreased the binding of bFGF to both HSPG and cell surface receptor sites.

**Effects of Elastase on bFGF Receptor Expression**—One means by which elastase could affect bFGF receptor binding could be through physical degradation or release of the cell surface receptor protein. To determine whether elastase treatment caused the direct loss of FGF receptors from the cell surface, whole cell lysates from control and elastase-treated cells were subjected to Western blot analysis using a monoclonal antibody generated against FGF receptor 1. No signifi-
cells were rinsed with ice-cold binding buffer, and 2 ng/ml 125I-bFGF was added for 2.5 h at 4°C. The cells were then washed three times with binding buffer and extracted with 1N sodium hydroxide. Plotted values represent the means ± S.E. where n = 4 for three separate binding experiments. Nonspecific binding (not competed by 50 μg/ml unlabeled bFGF) was subtracted from all the data.

A significant difference in the relative amounts of FGF receptor 1 protein was observed in control and elastase-treated cells (Fig. 7). These findings were confirmed by fluorescence microscopic analysis (data not shown). Thus, whereas elastase directly removes HSPG, it does not appear to degrade FGF receptors. Therefore, the elastase-mediated decrease in bFGF binding to its receptors is likely a consequence of decreased receptor affinity in the absence of HSPG (23).

DISCUSSION

The basic biological features underlying the pathological development and progression of chronic pulmonary obstructive diseases are poorly understood. As one component of these processes, we previously identified basic fibroblast growth factor as a product released by elastase digestion of pulmonary fibroblast cell matrices and documented its potent, negative regulatory effects on elastin gene transcription (12). In the present study, we expand our original findings to address the mechanism of elastase-mediated bFGF release. In this study, we have shown that elastase exposure effectively removes heparan sulfate chains from the cellular surface of pulmonary fibroblasts without an appreciable loss of bFGF receptor protein. Our findings also indicate that elastase exposure causes a concomitant decrease in the binding of bFGF at both HSPG and FGF receptor sites. In the absence of a significant effect at the FGF receptor level, we propose that the mechanism by which elastase decreases bFGF binding to its receptor is through disruption of the complex that bFGF forms with its receptor and the HSPG co-receptor. Based on the loss of available HSPG sites, the affinity of bFGF for its receptor is effectively reduced, which results in a net loss of bFGF binding to its receptor. These findings support previous work from our laboratory that demonstrated that HSPGs enhance bFGF activity by altering the affinity of FGF receptors without a direct consequence on FGF-stimulated signal transduction pathways (23, 28).

Release of HSPG by elastase from the extracellular matrix would be expected to have significant effects upon bFGF-mediated repair processes. First, the elastase-mediated loss of HSPG sites would be expected to accelerate the rate of bFGF diffusion within the extracellular space. Previous work from our laboratory has demonstrated that HSPG binding plays a key role in restricting bFGF movement within the matrix (29). Based on these findings, in the absence of HSPG binding sites, we predict that bFGF could diffuse unimpeded through pulmonary fibroblast matrices away from the initial sites of damage. Free bFGF would not be subject to the same physical constraints that restrict the transport of bFGF-HSPG complexes within the extracellular matrix. An enhanced rate of diffusion and bioavailability of bFGF due to the loss of HSPG sites may account in part for some of our previous findings in which bFGF elicited differential effects on elastin gene transcription at immediate and distant sites of elastolytic injury (12). Additional studies are necessary to address elastase-mediated changes in bFGF diffusivity within the extracellular environment.

In the present study, the observed release of HSPG by elastase is in good agreement with the work of van de Lest et al. (30), who demonstrated that proteoglycans are target molecules for elastase instilled in vivo. In both studies, elastase exposure resulted in incomplete cleavage of the core protein with significant loss of heparan sulfate chains. The differences in these two studies may be reflective of differences in heparan sulfate core protein expression in a discrete fibroblast population in vitro and a complex cellular population in vivo. In addition, recent work by van Kuppevelt et al. (31) has indicated that inhibition of proteoglycan synthesis in rat lung by a single instillation of β-D-xyloside resulted in an increase in free GAG in bronchoalveolar lavage fluid that was accompanied by alve-
Pulmonary fibroblast cultures were treated with buffer alone (lane 1) or with 0.5 μg/ml elastase (lane 2) for 15 min at 37 °C. Control and elastase-treated cell layers were subjected to SDS-PAGE and Western blot analysis. The blot was probed using a monoclonal antibody raised against FGF receptor 1 protein (clone 19B2; 1 μg/ml), followed by the addition of 0.5 μg/ml horseradish peroxidase-linked anti-mouse IgG secondary antibody before chemiluminescence detection. Increasing concentrations of control and elastase-treated cell lysates were subjected to SDS-PAGE and Western blot analysis to ensure linearity of the chemiluminescence signal, and the blot was stripped and reprobed using an antibody to β-tubulin to assess variations in protein concentration and electrophoretic transfer efficiency (data not shown).

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In summary, we have measured elastase-mediated heparan sulfate release from pulmonary fibroblast cultures and characterized the consequent effects on basic fibroblast growth factor receptor binding. We have found that under conditions that promote the release of bFGF from the matrix, there is a coincident release of HS and HSPG core proteins without an appreciable loss of bFGF receptor protein. These results suggest that the mechanism by which elastase decreased bFGF binding is through destabilization of the bFGF-heparan growth factor receptor-HSPG ternary complex following proteolytic cleavage of HSPG. Whereas bFGF is still able to bind to its receptor and initiate the appropriate signaling responses after elastase exposure, our data suggest a regulatory function for HSPG in modulating the activity of bFGF and other heparin binding growth factors within the lung under acute and chronic elastase exposure conditions.

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