Epithelial expression and release of FGF-2 from heparan sulphate binding sites in bronchial tissue in asthma

J K Shute, N Solic, J Shimizu, W McConnell, A E Redington, P H Howarth

Background: The most characteristic structural change evident in endobronchial biopsies in asthma, even in mild disease, is subepithelial collagen deposition within the lamina reticularis. This has been associated with progressive loss of lung function and the persistence of airway hyperresponsiveness, and has been linked to airflow fibroblast proliferation. A potent fibroproliferative factor in bronchoalveolar lavage fluid in asthma is fibroblast growth factor-2 (FGF-2). FGF-2 is a member of a family of heparin binding growth factors that bind to heparan sulphate proteoglycans (HSPG), an important determinant of FGF-2 activity. This study compared the level of expression and distribution of FGF-2 in relation to HSPG in bronchial tissue from normal and asthmatic subjects.

Methods: The distribution of FGF-2 and HSPG in intact and cleaved forms in endobronchial biopsies from normal and asthmatic subjects was examined using an immunohistochemical approach. A novel ELISA based method was developed to detect solubilisation of FGF-2 following addition of heparin and heparitinase to bronchial tissue slices.

Results: Immunohistochemical analysis showed that FGF-2 was co-localised to HSPG in epithelial and endothelial basement membranes. Epithelial FGF-2, but not HSPG, was significantly more abundant in patients with mild asthma than in normal subjects. In vitro experiments indicated that FGF-2 was released from binding sites in the tissue by heparin and heparitinase I.

Conclusions: FGF-2 is bound by HSPG in bronchial tissue. The most cell, through the release of heparin and endoglycosidase, may make a unique contribution to tissue remodelling in allergic asthma.
(0.34–14.5) mg/ml and four atopic non-asthmatic control subjects (all male) aged 26.5 (3.8) years with FEV1 100.3 (2.9)% predicted. Asthmatic subjects were mildly symptomatic and were receiving inhaled salbutamol as required as their only treatment. All non-asthmatic control subjects had histamine PC20 measurements outside the asthmatic range (>32 mg/ml).

The study was approved by the combined Southampton Hospitals and University Research ethics committee and all subjects gave written informed consent.

Fibreoptic bronchoscopy was performed in accordance with our previously published protocol and the recommendations of the National Heart, Lung, and Blood Institute. Bronchial biopsies (1–2 mm diameter) were obtained from third or fourth generation airway carinae and immediately either fixed for immunohistochemistry in ice cold acetone containing the protease inhibitors iodoacetamide (20 mM) and phenyl methyl sulphonyl fluoride (2 mM) or snap frozen in liquid nitrogen for tissue slice experiments.

Immunohistochemistry

Specimens for immunohistochemistry were processed into glycolmethacrylate (GMA) resin as previously described. Thin (2 μm) sections were cut from GMA embedded tissue, floated on 0.2% (v/v) ammonia in double distilled water, and collected onto poly-l-lysine coated glass slides. Sections were dried for 1 hour at room temperature and wrapped in aluminium foil for storage at 20°C.

Immunohistochemical staining was carried out using our published protocol. Endogenous peroxidase activity was inhibited by applying a solution of 0.3% hydrogen peroxide in 0.1% sodium azide for 30 minutes, and non-specific binding was blocked by applying a solution of 10% fetal bovine serum plus 1% BSA in Dulbecco’s modified Eagle’s medium (GibCo, Paisley, UK). All washes were in 0.05 M Tris buffered saline, plus 1% BSA in Dulbecco’s modified Eagle’s medium (GibCo, Paisley, UK). A detailed characterisation of the properties of these monoclonal anti-HS (10E4) which recognises intact HS, and a paired control antibody (Sigma Chemical Co, Poole, UK) were applied at 1:300 dilution for 2 hours. Streptavidin-biotin-peroxidase complex (Dako) was applied for 2 hours to amplify detection and aminoethyl carbazole (93 mg/ml) was added as the chromogen. Sections were counterstained with Mayer’s haematoxylin and blued in cold 1% aqueous aluminium foil for storage at 20°C.

To measure the basal and stimulated release of FGF-2 from bronchial tissue, a novel one step method for the rapid capture, detection, and quantitation of the growth factor was developed. Tissue slices (20 μm cryosections) were cut from frozen biopsies embedded in OCT mounting medium (Tissue Tek II, Miles Laboratories Inc, Naperville, USA) and placed in 200 μl cold RPMI medium (GibCo) in the wells of a 96-well microtitre plate precoated with anti-FGF-2 (R&D Systems, Oxford, UK). Plates were warmed to room temperature before adding stimuli. Heparin from pig intestinal mucosa with molecular weight 13 500–15 000 (Calbiochem, Nottingham, UK), the HS degrading endoglycosidase heparitinase I (Seikagaku Corp, MMP-3 (Biogenesis), or streptokinase (Sigma Chemical Co) were added at the final concentrations and incubated for 10, 15, 20, 40, and 60 minutes at 37°C. FGF-2 standards were included at each time point because the sensitivity of the ELISA is reduced at time points less than 60 minutes. Incubations were carried out in duplicate using non-adjacent sections. To test for protein dependent release of FGF-2, 100 μg/ml 2-macroglobulin was added in some experiments. Following incubation, tissue and medium were removed, the wells washed, and FGF-2 that had been released and captured by the primary antibody was quantitated by completing the subsequent ELISA steps according to the manufacturer’s instructions.

Statistical analysis

Clinical data for age and FEV1 % predicted were expressed as mean (SE) and for PC20 as geometric mean (range). Immunohistochemical data were expressed as median (range) and compared using the Mann-Whitney U test and the Wilcoxon test for unpaired and paired data, respectively. FGF-2 concentrations in culture supernatants were expressed as mean (SE). Release of FGF-2, expressed as percentage change from baseline, was analysed using a repeated measures ANOVA model with a post hoc Fisher’s PLSD test for concentration-response experiments, an unpaired t test to compare tissue from asthmatic and non-asthmatic subjects, and a paired t test to study the effect of 2-macroglobulin. Analysis was performed using StatView 5.01 for Macintosh computers (Abacus Concepts, Berkeley, CA, USA). A significance level of 5% was accepted.

RESULTS

Intracellular and extracellular FGF-2 immunoreactivity was detected in bronchial tissue from control (fig 1A) and asthmatic subjects. Intracellular FGF-2 was observed within bronchial epithelial cells and in cells within the subepithelial region. Extracellular FGF-2 was seen in the pericellular matrix of endothelial cells and in the epithelial basement membrane. The intact form of HS was detected in a linear distribution on epithelial and endothelial basement membranes where it was found in abundance in both control (fig 1B) and asthmatic tissue. Intracellular intact HS was detected only in endothelial cells and could be detected at both lumenal and basal aspects of these cells. The cleaved form of HS was also found predominantly in endothelial basement membranes and, notably in the asthmatics, in the extracellular matrix of these cells.
many large mononuclear cells in the tissue (fig 1C). However, these same cell types did not stain for intact HS. No immunoreactivity was detected if the primary antibody was replaced with an isotype matched control mouse IgG1 (fig 1D) or if the primary antibody was omitted (not shown).

Quantitative analysis indicated a greater area of epithelial FGF-2 immunostaining in asthmatic tissue than in control tissue (median 8.7% vs 5.3%, p < 0.05; fig 2). However, there was no significant difference in the proportion of blood vessels staining positively for FGF-2 between these two subject groups (median 36% vs 29.5%, p = 0.89). The cleaved form of HS was significantly less abundant than the intact form at the endothelial level and in the normal epithelial basement membrane (table 1). The proportion of epithelial basement membrane and the proportion of blood vessels staining positively for both intact and cleaved forms of HS did not differ significantly between the two subject groups (table 1).

Release of FGF-2 from bronchial tissue

In preliminary experiments FGF-2 was undetectable in the supernatants of bronchial tissue slices incubated in tissue culture plastic ware with or without stimuli. We suspected that this was due to rebinding of FGF-2 to tissue sites. Therefore, to prevent rebinding, tissue slices were incubated directly in the wells of ELISA plates in which the specific high affinity primary antibody used to coat the wells would effectively compete with tissue binding sites and capture the released growth factor. Using this assay we investigated the effect of substances that might influence the binding of FGF-2 to HS side chains.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Quantitation of immunohistochemical staining for intact and cleaved forms of heparan sulphate (HS) in bronchial tissue from seven asthmatic subjects and six non-asthmatic control subjects</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Intact HS</td>
</tr>
<tr>
<td>% blood vessels</td>
<td></td>
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<tr>
<td>Asthma</td>
<td>100 (28.2–100)</td>
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<tr>
<td>Control</td>
<td>95.3 (77–100)</td>
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<tr>
<td>% epithelial basement membrane</td>
<td></td>
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<tr>
<td>Asthma</td>
<td>19.9 (0–46.7)</td>
</tr>
<tr>
<td>Control</td>
<td>10.9 (0.36–35.9)</td>
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</table>

Data shown as median (range).

*Immunostaining for the cleaved form of HS significantly (p < 0.05) less extensive than for the intact form.

Figure 1 Representative immunohistochemical staining patterns. (A) Anti-FGF-2 in tissue from a control subject. (B) Antibody 10E4 (to identify intact heparan sulphate (HS)) in tissue from a control subject. (C) Antibody 3G10 (to identify cleaved HS) in tissue from an asthmatic subject. (D) Control section stained with an IgG1 isotype control antibody. Black arrows indicate epithelial basement membrane, black arrowheads indicate epithelium, red arrows indicate endothelium, and blue arrows indicate positively staining cells in the submucosa. Scale bar = 50 μm.

Figure 2 Quantitation of FGF-2 immunostaining in the bronchial epithelium (circles) and vascular endothelium (diamonds) in asthmatic and non-asthmatic subjects. Horizontal bars represent median values. Between group comparisons were performed using the Mann-Whitney U test.
Heparin
No significant effect was observed at incubation times of 10, 15, 20, or 40 minutes at any concentration of heparin (n = 4, results not shown). At an incubation time of 60 minutes (n = 6) there was a concentration-dependent increase in FGF-2 release (fig 3A). This effect was of borderline significance (p = 0.055) at a heparin concentration of 10 µg/ml. At 100 µg/ml heparin there was a highly significant (p = 0.0001) effect with FGF-2 levels increased to a mean of 217% (95% CI 140 to 293) of baseline—that is, from 29.1 (6.4) pg/ml to 58.7 (12.5) pg/ml. There was no further increase in FGF-2 release at a heparin concentration of 200 µg/ml (52.7 (17.1) pg/ml, n = 3).

Additional experiments were performed at optimal conditions (heparin 100 µg/ml for 60 minutes) to compare tissue slices from atopic asthmatic donors (n = 5) and atopic non-asthmatic donors (n = 4) and to study the effect of the protease inhibitor α2-macroglobulin (n = 3). There was a trend for a greater response in tissue from the asthmatic group (mean 237% v 171% of baseline), but this difference did not reach statistical significance (p = 0.11). Inclusion of α2-macroglobulin did not significantly alter unstimulated release of FGF-2 (60.8 (14.4) pg/ml v 44.9 (9.5) pg/ml, p = 0.44) and did not inhibit release induced by heparin (fig 4).

Heparitinase I
No significant effect of heparitinase I at any concentration was observed at incubation times of 10, 15, 20, or 40 minutes (n = 4, results not shown). At an incubation time of 60 minutes (n = 6) heparitinase I concentrations of 2.5 µU/ml and 12.5 µU/ml were without effect but 25 µU/ml heparitinase I significantly (p = 0.0048) increased FGF-2 levels to a mean of 249% (95% CI 102 to 397) of baseline—that is, from 37.4 (13.4) pg/ml to 70.7 (18.21) pg/ml (fig 3B).

Additional experiments, as described above for heparin, were performed with heparitinase 25 µU/ml for 60 minutes. There was no significant difference in the magnitude of the response between tissue from asthmatic and non-asthmatic subjects (mean 237% v 195% baseline, p = 0.64). The effect of heparitinase I was not inhibited by the protease inhibitor α2-macroglobulin at 100 µg/ml (fig 4).

Matrix metalloproteinase-3 (MMP-3, stromelysin) and streptokinase
For comparison, the effects of enzymes that might lead to cleavage of proteoglycan core proteins—either directly or indirectly via activation of other proteases—were investigated. Incubation of tissue slices with MMP-3 (130 ng/ml) or streptokinase (500 IU/ml) for 60 minutes did not increase the release of FGF-2.

DISCUSSION
This study describes the localisation and relative abundance of immunoreactive FGF-2 and HS in bronchial tissue from subjects with mild asthma and healthy control subjects. We have shown for the first time that epithelial FGF-2 immunostaining is increased in asthmatic tissue. HS was present in epithelial basement membranes and at vascular sites, but quantitative analysis revealed no detectable difference between the subject groups at these locations. We have also described a novel method to detect release of FGF-2 from bronchial tissue slices and found that heparin and heparitinase I induce FGF-2 release in a time and concentration dependent manner.

We have previously identified higher basal levels of FGF-2 in BAL fluid in patients with atopic asthma.27 In principle, this could represent increased generation and release or, alternatively, decreased catabolism. The higher baseline levels in BAL fluid may reflect the increased expression of FGF-2 by epithelial cells in asthma that we have shown in this study. A recent report has also indicated increased FGF-2 expression in asthmatic subjects by cells in the submucosa which were identified in order of abundance as CD34+ cells > eosinophils > macrophages > T cells > mast cells.28 FGF-2 is a mitogen for many cells including fibroblasts and endothelial cells—cell types that express CD34—and these cells may therefore be both a source and a target of FGF-2 activity.

The co-localisation of FGF-2 and HS in bronchial tissue suggests that FGF-2 is largely bound in an inactive form on
the endothelium and in epithelial basement membranes, providing an extracellular reservoir of the growth factor. FGF-2 binds to the HS side chains of HSPG, the major pericellular HSPGs being perlecan in the basement membrane and the syndecans and glypicans of the cell surface. Because cleavage of HS side chains may indicate local release of bound growth factor, we studied the expression of HS using two specific antibodies—one which recognises intact HS chains and one which recognises an epitope generated by heparitinase digestion of HSPG. Intact HS was the predominant form in both normal and asthmatic tissue. We had anticipated that cleaved HS might be more abundant in asthmatic tissue but, in fact, we could find no significant difference between the subject groups in the distribution or abundance of either form of HS at epithelial and endothelial sites. However, the variability of measurements and the sample size may have limited the ability of the study to detect such a difference. In contrast, a recent report indicates increased expression of perlecan, biglycan, and small HSPGs by fibroblasts from subjects with mild asthma. The mononuclear cells that stained for cleaved but not intact HS in asthmatic tissue in the present study may have been monocytes/macrophages—cells that express HS on maturation in tissues but not in the blood and constitutively release HS degrading activity on contact with tissue matrices.

We previously speculated that the significant increase in FGF-2 in the BAL fluid following endobronchial allergen challenge, which occurs within 10 minutes and is therefore too early to reflect de novo synthesis, may have reflected the release of preformed FGF-2 from storage sites in bronchial tissue in response to enzymatic cleavage of HS or proteoglycan displacement. Because the rapid in vivo release of FGF-2 occurred in temporal association with mast cell degranulation, we investigated the effects of heparin and heparitinase I on FGF-2 release using an ex vivo tissue slice model.

Heparin has an important impact on FGF-2 activity. A recent study has indicated dual functions of heparin in the regulation of this activity: cell surface bound heparin potentiates FGF-2 receptor interactions whereas soluble heparin binds FGF-2 in solution and inhibits its interaction with specific cell surface receptors. Heparin may therefore either stimulate or inhibit FGF-2 receptor binding and bioactivity, the net effect being dependent on the concentration of heparin, the number of HS sites on the target cell, and the affinity and number of binding sites for heparin on the target cell.

In the present study we have shown that heparin stimulates the release of FGF-2 from bronchial tissue slices. The concentration of heparin required (100 μg/ml) was higher than that reported to displace FGF-2 from isolated bovine corneal basement membrane (10 μg/ml) and from the subendothelial extracellular matrix produced by cultured endothelial cells (5 μg/ml), perhaps reflecting the greater number of non-specific heparin binding sites in whole tissue. However, the effective concentration of heparin was of the order of that previously estimated to be released by sensitised human lung tissue fragments after allergen challenge (mean approximately 19.3 μg/g tissue), although concentrations of heparin achieved locally in the immediate vicinity of mast cells after release are presumably much higher. Although we previously detected FGF-2 release into BAL fluid within 10 minutes of allergen exposure, release from tissue slices in the present study was not evident at incubation times shorter than 60 minutes. The reason for this difference between the in vivo and in vitro kinetics of FGF-2 release is unclear. Enzymes that cleave HSPGs—either their core proteins or GAG side chains—are likely to influence the form and function of FGF-2. For example, the bacterial endoglycosidase heparitinase I has been shown to reduce FGF-2 binding to fibroblasts and to abrogate FGF-2 induced proliferation through cleavage of the cell surface HSPG syndecan-4. Kato et al recently identified a novel pathway for the regulation of FGF-2 activity involving another cell surface HSPG syndecan-1. Under physiological conditions the soluble syndecan-1 ectodomain potently inhibits heparin induced FGF-2 mitogenicity. However, partial degradation of the GAG chains by bacterial heparitinase or platelet heparanase, as may occur at sites of inflammation and tissue injury, converts syndecan-1 to an FGF-2 activator by generating stimulatory oligosaccharide sequences. The activity of FGF-2 at cell surfaces may therefore be regulated by the shedding and limited degradation of HSPGs. In the present study, extracellular FGF-2 was immunolocalised to the luminal and basal surfaces of endothelial cells and to the epithelial basement membrane. Release of FGF-2 following incubation of bronchial tissue with heparitinase I confirms that the growth factor was bound to HS side chains of HSPG and indicates a potential mechanism for FGF-2 release at sites of allergic inflammation. Plasminogen activators, acting directly or as activators of a number of MMPs, have been implicated in the release of FGF-2 from endothelial cells. At the concentrations tested, however, we were unable to demonstrate any effect of the plasminogen activator streptokinase or of the proteoglycanase MMP-3 on FGF-2 release. The same nanomolar concentration of MMP-3 was previously shown in an in vitro model to induce damage and loss of GAGs from gut mucosal tissue, an effect that could be inhibited with 2-macroglobulin. The lack of effect of 2-macroglobulin at the same concentration in our study suggests that heparin and heparitinase I were acting directly on the GAG side chains of tissue HSPG.

The studies reported here link an aspect of airway inflammation in asthma—namely, mast cell activation—with an aspect relevant to the remodelling process in this disease—namely, FGF-2 release. Activation of FGF-2 by release from sites of encryption is likely to contribute to the structural changes seen in the asthmatic airway, including fibroblast proliferation and angiogenesis. The modulation of the activity of this and other growth factors—such as transforming growth factor β (TGF-β) and platelet-derived growth factor (PDGF)—by proteoglycan binding represents an important post-translational regulatory process and indicates that mechanisms relevant to the chronic structural airway changes can occur in parallel with the inflammatory process. A better understanding of other factors involved in post-translational regulation of growth factor bioavailability and the influence of airway anti-inflammatory therapy on these processes is thus warranted.

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