Effect of heparin on pulmonary fibroblasts and vascular cells

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Abstract
Background There is a large increase in mast cell numbers in fibrotic lung tissue, suggesting that mast cells may play a part in the pathogenesis of pulmonary fibrosis. Glycosaminoglycans, such as heparan sulphate, that are structurally related to heparin (a mast cell product) are part of the extracellular matrix and known to regulate cell growth. Basic fibroblast growth factor is a heparin binding growth factor produced by endothelial cells.

Methods A study was carried out to examine the effect of heparin, basic fibroblast growth factor, and mast cell products on the proliferation of normal human lung fibroblasts and the effect of adding heparin on the proliferation of lung fibroblasts and pulmonary vascular cells incubated with basic fibroblast growth factor.

Results Heparin at low concentration (0.03, 0.3–1.0 μg/ml) stimulated the proliferation of normal human lung fibroblasts in culture whereas a higher concentration (100 μg/ml) had an inhibitory effect. Mast cell products also stimulated the proliferation of fibroblasts, and the effect was decreased by pretreatment with heparinase or protamine. Heparin enhanced the growth of both fibroblasts and pulmonary vascular cells induced by low concentrations of basic fibroblast growth factor.

Conclusions Mast cells in fibrotic lung tissue may regulate fibroblast proliferation by releasing heparin. These results suggest that endothelial cells may interact with mast cells and modulate fibroblast growth by release of basic fibroblast growth factor.

Heparin is released into the extracellular space from mast cell granules through both immunological and non-immunological activation of the mast cell. Heparin released from mast cells at the site of angiogenesis stimulates endothelial cell migration, and has very complex biological actions, including inhibition of coagulation, regulation of complement expression, and inhibition of smooth muscle cell proliferation.

A substantial increase in mast cell numbers is observed in fibrotic lung tissue, leading to the suggestion that mast cells may have a role in the pathogenesis of pulmonary fibrosis. To determine the role of heparin, we investigated the direct effect of heparin with and without basic fibroblast growth factor on pulmonary fibroblast proliferation, as basic fibroblast growth factor is produced by endothelial cells and is recognised as a heparin binding growth factor. We also examined the effects of heparin and basic fibroblast growth factor on bovine pulmonary vascular cells, because vascular changes are often observed in connective tissue disorders.

Methods

MATERIALS
Fetal bovine serum (FBS) was obtained from Bockneck Laboratories Inc (Canada), heat inactivated at 56°C for 30 minutes, and

![Figure 1](http://thorax.bmj.com) Phase contrast micrographs: (A) normal human diploid fibroblast (TIG); (B) bovine pulmonary artery endothelial cells (PACE); (C) bovine pulmonary artery smooth muscle cells (CSM).
Effect of heparin on pulmonary fibroblasts and vascular cells

Figure 2 Dose-response relationships of (A) basic fibroblast growth factor (bFGF); (B) heparin; (C) heparin in the presence of bFGF 0·01 ng/ml (■) and 0·3 ng/ml (●) with regard to modulation of normal lung fibroblast proliferation. Data are expressed as mean cell counts with standard errors. Similar results were obtained in three independent experiments.

checked for its growth activity. Heparin and protamine sulphate were obtained from Sigma (St Louis, Missouri) and heparinase from Seikagaku Kogyo Company, Ltd (Tokyo). Recombinant human basic fibroblast growth factor was a generous gift from Takeda Pharmaceutical Company Ltd (Osaka), and Dulbecco’s modified Eagle medium (DME) was from Sigma. Most of the other reagents were of special grade and manufactured by Wako Pure Chemical Industries Ltd (Osaka).

CELL LINES

Fibroblasts Normal diploid fibroblasts were derived from a human lung as described.17

Pulmonary vascular cells Fresh pulmonary artery dissected from a one month old calf was rinsed in Eagle medium, cut open lengthwise, and placed on a sterile Teflon sheet with the intimal side up. Endothelial cells were gently scraped off the intimal surface with a surgical blade, suspended in Eagle medium with 10% fetal bovine serum in a Corning tissue culture dish, and cultured at 37°C in a humidified incubator in a 95:5 (air:carbon dioxide) atmosphere. After the endothelium had been completely removed, the medial layer of the pulmonary artery was dissected with fine forceps, cut into 1 mm² fragments, and placed in plastic dishes. Once these fragments were adhering to the surface of the dish the growth medium was gently added to the culture. Smooth muscle cells were obtained by outgrowth from the explants of medial layer fragments. Routine subcultivations were performed at a 1:8 split ratio once every five days with one intervening change of medium. Cells were counted with a haemocytometer and Coulter counter (Coulter Electronics Inc, Hialeah, Florida) at each subcultivation. Endothelial cells and smooth muscle cells were identified by their characteristic microscopic morphology (fig 1). Endothelial cells were also examined for expression of factor VIII related antigen.

ISOLATION OF MAST CELLS

Mast cells were collected from the peritoneal cavities of normal Wistar rats weighing 150–250 g by a slightly modified version of the method described by Suzuki et al.18 The cells were suspended in Tyrode’s solution (137 mM NaCl, 2·7 mM potassium chloride, 1·0 mM manganese chloride, 1·8 mM calcium chloride, 0·4 mM NaH₂PO₄, 11·9 mM NaHCO₃, and 5·6 mM glucose) and layered on bovine serum albumin-saline solution, the density of which was adjusted to precisely 1·068 and the pH to 7·0. After centrifugation at 100 g at 4°C for 15 minutes the bottom layer, containing mast cells, was washed twice with 5 ml of 0·1% bovine serum albumin in Tyrode’s solution and suspended in the same medium at about 10⁶ cells/ml. The purity of mast cells was about 80–90% when observed with an Olympus phase contrast microscope (Olympus Optical Company, Tokyo).
RELEASE OF MEDIATORS
Samples of the mast cell suspension (1 × 10^6 cells/ml) were incubated for 15 minutes with and without compound 48/80 (10 μg/ml) at 37°C. The mixtures were centrifuged at 1500 g for five minutes at 4°C, and the supernatant was used for culture after microfiltration with a 0.22 μm filter.

CELL GROWTH ASSAY
Pulmonary vascular cells
Cells in the growth phase were seeded at a density of 4 × 10^5 cells/cm² into each well of 24 well plates (Falcon) containing 1 ml of assay medium (Eagle’s medium supplemented with 10% fetal bovine serum) and cultured for 24 hours. Then the cells were supplied with fresh assay medium containing basic fibroblast growth factor or heparin, or both, at varying concentrations. The cells were allowed to proliferate at 37°C for 72 hours. After incubation cells were removed by trypsinisation and suspended by pipetting; cell numbers were counted with a Coulter counter. The numbers were similar to those determined with a haemocytometer and no cell aggregates were observed. Results were obtained from triplicate samples and expressed as mean values with standard errors.

Fibroblasts
Fibroblasts in the growth phase were seeded at a density of 4 × 10^5 cells/cm² in each well containing 1 ml of assay medium (Eagle’s medium supplemented with 1% fetal bovine serum) and cultured for 24 hours. The medium was then changed to a fresh assay medium containing basic fibroblast growth factor or heparin, or both, at varying concentrations or to a test medium containing mast cell products, which were pretreated with specific heparin inhibitors, heparinase (0.05 unit/ml) and protamine (200 μg/ml), or with Tyrode’s solution. The amount of supernatant from mast cells to be added to the medium was determined by a preliminary assay to obtain the maximum effect on fibroblast proliferation. As control media, we used Eagle’s medium with 1% fetal bovine serum alone and with 1% fetal bovine serum containing supernatants of mast cell suspension that had not been stimulated with compound 48/80. Each supernatant was also pretreated with heparinase and protamine or Tyrode’s solution. The cells were allowed to proliferate for 72 hours with these test media and removed by trypsinisation. Cells were counted with a Coulter counter. Results were obtained from quadruplicate samples and expressed as means with standard errors.

ANALYSIS
One way analysis of variance and Bonferoni’s method were used for statistical analysis.
Results

EFFECT OF BASIC FIBROBLAST GROWTH FACTOR AND HEPARIN ON FIBROBLASTS, ENDOTHELIAL CELLS, AND VASCULAR SMOOTH MUSCLE

Lung fibroblasts and endothelial cells were stimulated by basic fibroblast growth factor in a dose dependent manner \((p < 0.05)\) (fig 2A and 3A). Heparin appeared to cause a biphasic response on the growth of lung fibroblasts, causing stimulation at concentrations of 0-03 and 0-3-1 \(\mu\)g/ml \((p < 0.05)\) but a reduced effect at 0.04-0.1 \(\mu\)g/ml and at the highest concentration \((>3 \mu\)g/ml\) (fig 2B). In the presence of basic fibroblast growth factor, heparin enhanced fibroblast growth in a similar manner \((p < 0.05)\) (fig 2C).

Endothelial cell proliferation showed a dose related response to basic fibroblast growth factor but was suppressed by heparin \((p < 0.05)\) (fig 3). Growth promotion by basic fibroblast growth factor was enhanced by low concentrations of heparin, however \((0.1 \mu\)g/ml\) \((p < 0.05)\) (fig 3C).

Vascular smooth muscle cell growth (fig 3) was not affected by basic fibroblast growth factor or heparin, though heparin \((0.5 \mu\)g/ml\) stimulated the growth of smooth muscle cells in the presence of basic fibroblast growth factor \((p < 0.05)\).

Discussion

An increase in mast cells has been reported in patients with lung fibrosis.\(^{10-12}\) As the interstitial fibroblast is an important cell in the development of fibrotic lesions, the products of mast cells, such as histamine, may interact with fibroblasts and stimulate their replication, as reported by Jordana et al.\(^{19}\)

We have shown that glycosaminoglycans such as heparan sulphate, heparin, and hyaluronic play an important part in regulating cell proliferation.\(^{20,21}\) Mast cells are the most important storage site for heparin in many mammalian tissues.\(^{22}\) Rao et al reported that rat fibroblasts phagocytosed rat mast cell granules and suggested that alterations in fibroblast behaviour occurred as a consequence of this process.\(^{23}\) Cultured rat and human fibroblasts ingest and degrade the heparin matrix of mast cell granules.\(^{24}\) We therefore examined the direct effect of heparin on lung fibroblast proliferation.

In the present study stimulation of cell proliferation was observed in cultures of normal human lung fibroblasts exposed to heparin at concentrations of 0.03 as well as 0.3-1 \(\mu\)g/ml. How heparin modulates the fibroblast proliferation on a molecular basis remains to be determined. Norby reported that heparin at concentrations of 0.2-400 IU (1.5 \(\mu\)g and 3.08 mg)/ml growth medium stimulated cell multiplication, though the extent of the stimulation produced by various concentrations of heparin varied in different cell lines.\(^{25}\)

Fibroblast proliferation occurred in response to mast cell products in this study. As heparinase and protamine decreased this activity, native heparin from mast cells is likely to interact with and promote fibroblast proliferation. There was no significant difference in growth stimulation between the supernatant from stimulated mast cells pretreated with heparin inhibitors and other controls at the concentration we used. As mast cells possess other fibroblast stimulating factors, such as histamine,\(^{19}\) however, the supernatant from stimulated mast cells might promote fibroblast growth at different concen-
trations despite pretreatment with heparin inhibitors. Basic fibroblast growth factor stimulated fibroblast proliferation in a dose dependent manner as previously reported.26 In the presence of basic fibroblast growth factor heparin enhanced the growth promotion of fibroblasts. As heparin has been shown to protect basic fibroblast growth factor from degradation,27 heparin could be having an indirect effect through basic fibroblast growth factor.

Proliferation of pulmonary artery endothelial cells was stimulated by basic fibroblast growth factor and inhibited by heparin as reported previously.20 22 26 Molecular mechanisms of growth suppression by heparin have not yet been determined. As heparan sulfate, a molecule structurally related to heparin and produced by endothelial cells, is important in contact inhibition,29 possibly heparin has similar substrate-cell interactions. Pulmonary artery smooth muscle cells were not affected by basic fibroblast growth factor or heparin. Other workers have reported a stimulant effect of basic fibroblast growth factor and an inhibitory effect of heparin on smooth muscle cells9 8 derived from various vessels at different sites. The conflicting results in vitro are often explained by tissues or species specificity.

Heparin enhanced the growth promotion by basic fibroblast growth factor of pulmonary artery endothelial cells and smooth muscle cells in this study. Heparin is known to increase or stabilize the biological activity of acidic fibroblast growth factor30 through protection from proteolytic inactivation and conformational change.31 32 Heparin also protects the biological activity of basic fibroblast growth factor from denaturation. There have been reports that growth promotion by basic fibroblast growth factor was suppressed by heparin.22 25 As the concentration of heparin in those reports is high, such as 50 or 100 μg/ml, we consider that heparin potentiates the stimulatory effect of basic fibroblast growth factor on the proliferation of endothelial cells and smooth muscle cells at low concentration, while the inhibitory effect of heparin predominates at high concentration.

Lung fibrosis is often associated with a collagen vascular disease,33 and the existence of an endothelial cell injury has been suggested.34 Lykke et al reported that mast cells lie adjacent to areas of focal hydropic degeneration of endothelial cells of venules of the microcirculation in some patients with diffuse interstitial pulmonary fibrosis.35 It has also been reported that endothelial cells synthesize basic fibroblast growth factor,36 though the nucleotide sequence of basic fibroblast growth factor complementary DNA indicates that the protein is synthesised without a signal sequence and lacks the structural features normally required for protein secretion.37 38 Heparan sulphate, released either directly by cells or through degradation of the extracellular matrix, seems to act as a carrier for basic fibroblast growth factor and facilitates the diffusion of locally produced growth factor by competing with its binding to surrounding matrix structures.39 40 Basic fibroblast growth factor is known to bind heparin to a greater extent than heparan sulphate.

Our in vitro studies suggest that mast cells and endothelial cells may interact and modulate fibroblast proliferation by release of heparin and basic fibroblast growth factor respectively. They also suggest that heparin from mast cells may stimulate the growth of vascular cells at certain concentrations in the presence of basic fibroblast growth factor whereas heparin alone inhibits the proliferation of endothelial cells.

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