Original Articles

Hepatocyte growth factor and neutrophil elastase in idiopathic pulmonary fibrosis

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It has been hypothesized that hepatocyte growth factor (HGF) may play an important role in regulating the growth of lung epithelium and in the regeneration of the lung as a paracrine or endocrine factor in idiopathic pulmonary fibrosis (IPF). Based on this background, serum HGF was measured in 31 IPF patients (21 male/10 female, median age 60 years). Fifteen age-matched normal non-smokers served as the control. Hepatocyte growth factor was measured by enzyme-linked immunosorbent assay with monoclonal and polyclonal antibodies against human HGF (Otsuka Assay Laboratories, Tokushima, Japan). Elastase: α1-proteinase complex was also measured by enzyme-linked immunosorbent assay. No patients had significant liver or renal dysfunction. As a result, mean (standard error) serum HGF concentration of the patients with IPF was 0.384 (0.022) ng ml⁻¹, which was significantly high compared to normal non-smokers (0.213 (0.012) ng ml⁻¹, P<0.001, 95% confidence interval was between 0.104 and 0.238]. Serum HGF values correlated strongly with the plasma elastase: α1-proteinase inhibitor complex (R=0.679, P<0.001). Immunohistochemical staining of lung tissue with anti-human neutrophil elastase showed scattered immunopositive cells mainly in interstitium. Immunohistochemical staining with mouse anti-human HGF antibody showed that HGF was distributed to the lung epithelial cells in IPF lung specimens obtained by open lung biopsy. These results suggest that HGF may play an important role in the pathogenesis of IPF.

Introduction

In the lung, hepatocyte growth factor (HGF) is synthesized and secreted by mesenchymal cells, such as macrophages, endothelial cells and fibroblasts, and controls proliferation and morphogenesis of epithelial cells (1). It has recently been revealed that the lung has an endocrine function and produces HGF for the regeneration of injured tissues or organs (2). Hepatocyte growth factor also acts as a pulmotrophic factor in lung regeneration after acute lung injury (3), and is a potential paracrine growth factor for rat alveolar type II cells in primary culture (1). Therefore, the authors hypothesized that HGF may play an important role in regulating the growth of lung epithelium and in the regeneration of the lung as a paracrine or endocrine factor in idiopathic pulmonary fibrosis (IPF). On the other hand, human neutrophil elastase is believed to play an important role in the pathogenesis of IPF with regard to degrading the lung interstitium (4). Based on this background, HGF was measured in serum, bronchoalveolar lavage fluid (BALF) and elastase: α1-proteinase inhibitor (PI) complexes in plasma and BALF in patients with IPF.

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Methods

ETHICAL APPROVAL AND METHOD OF DIAGNOSIS OF IPF

The protocols of this study were approved by the institutional review board for human studies, and informed written consent was obtained from the subjects. Serum and plasma samples were obtained from 31 patients with IPF. The diagnosis was made on clinical, radiological, physiological and histological grounds. The criteria used included: history of dyspnoea and cough, fine crackles on physical examination, compatible findings on the chest radiograph (diffuse basal reticulonodular shadowing), restrictive pattern of pulmonary function, and a reduced diffusing capacity. In addition, no associated connective tissue diseases were present nor was there a history of occupational exposure or hypersensitivity. Histological confirmation was obtained in all cases (transbronchial biopsy in 16 patients and open lung biopsy in 15 patients). In all patients, high-resolution computed radiographic scanning of the lungs (HRCT) was performed.

SUBJECTS

The study population consisted of 10 women and 21 men with an average age of 60 years. The 15 normal non-smokers, six women and nine men with an average age of 55 years, had no history of lung diseases and no clinical findings suggesting lung disease. They all had normal chest X-rays and their pulmonary function test results were within the normal range. Of 31 patients, 13 patients were receiving low-dose corticosteroids, and two patients were receiving low-dose corticosteroids as well as oral cyclophosphamide.

Blood samples with and without EDTA were obtained before breakfast. After centrifugation at 1000 g for 10 min at 4°C, the plasma and the serum was frozen and stored at -70°C until use.

BRONCHOALVEOLAR LAVAGE

To sample the lower respiratory tract, flexible fiberoptic bronchoscopy and bronchoalveolar lavage were performed in 15 patients with IPF and 15 normal non-smokers. Bronchoalveolar lavage was performed by infusing three 50-ml aliquots of sterile saline at the site of the anterior segment of the right lower lobe. The last two aliquots were saved for evaluation. Cells were separated from alveolar lavage fluid by centrifugation (300 g for 10 min). Total cell number and cell differential counts were also evaluated. Bronchoalveolar lavage fluid was frozen and stored at -70°C until use.

MEASUREMENT OF ELASTASE: α₁-PROTEINASE COMPLEX LEVELS IN PLASMA AND BALF

Elastase:α₁-proteinase (PI) complex concentration was determined by using an enzyme-A kit purchased from Merck (Diagnostica Merck, Darmstadt, Germany). Briefly, the plasma samples were added to wells coated with sheep anti-neutrophil elastase IgG. This antibody does not cross-react with cathepsin G or other neutrophil proteinases. After incubation and washing, the solid phase-bound elastase:α₁-proteinase complexes were further incubated with alkaline phosphatase-labelled rabbit anti-α₁-proteinase IgG. After further washes, p-nitrophenylphosphate was added to measure the amount of solid phase-bound complexes. The assay was calibrated using a standard solution of known elastase:α₁-proteinase complex concentration. The lower detection limit of this assay was 3 ng ml⁻¹. Data were expressed as mean values from duplicate determinations.

MEASUREMENT OF HGF LEVELS IN SERUM AND BALF

Hepatocyte growth factor was measured by ELISA with monoclonal and polyclonal antibodies against human HGF (Otsuka Assay Laboratories, Tokushima, Japan). This assay was calibrated using a standard solution of known HGF concentration. The lower detection limit of this assay was 0.02 ng ml⁻¹. Data were expressed as mean values from duplicate determinations.

IMMUNOSTAINING BY ANTI-NEUTROPHIL ELASTASE

To evaluate the site of neutrophil accumulation in patients with IPF, 15 specimens obtained by open lung biopsy were immunohistochemically stained using anti-human neutrophil elastase (DAKO, M752) by the Avidin-Biotin-Peroxidase (ABC) method using a DAKO LSAB kit (DAKO Japan, Kyoto). Normal lung was also stained as a control.

IMMUNOSTAINING BY ANTI-HGF ANTIBODY

Immunohistochemical staining with rabbit polyclonal anti-human HGF antibody (gift from Toshikazu Nakamura) was performed in 15 lung specimens by ABC method using a DAKO LSAB kit (DAKO Japan, Kyoto). Normal lung was also stained as a control.

STATISTICAL METHODS

All comparisons between groups were made using the non-parametric Wilcoxon–Mann–Whitney rank order test. Correlations were evaluated by the
Results

Figure 1 (a) shows the concentrations of plasma elastase: α1-proteinase complex in the study population. Data are expressed as means ± standard error. Plasma elastase:α1-proteinase complexes in patients with interstitial pneumonia were significantly high (539.9 ± 77.4 ng ml⁻¹) compared with normal non-smokers (130.3 ± 5.5 ng ml⁻¹, P<0.01, 95% confidence interval was between 183.9 and 635.3).

Figure 1 (b) shows the concentrations of serum hepatocyte growth factor (HGF) in the study population. Data are expressed as means ± standard error. Serum HGF in patients with IPF were significantly high (0.384 ± 0.022 ng ml⁻¹) compared with normal non-smokers (0.213 ± 0.014 ng ml⁻¹, P<0.01, 95% confidence interval was between 0.104 and 0.238).

In BALF obtained from 15 patients with IPF, the total cell count was 1.85 ± 0.23 × 10⁵ ml⁻¹, and differential counts of macrophages, lymphocytes, neutrophils and eosinophils were 81.0 ± 6.4%, 15.3 ± 6.1%, 3.0 ± 1.1% and 0.7 ± 0.4%, respectively.

Figure 2 (a) shows the concentrations of elastase:α1-proteinase complex in BALF in the study population. As the mean concentration of total protein in BALF in both study groups was almost the same, data (means ± standard error) were expressed as the absolute values. Complexes in BALF in patients with IPF were significantly high (213.3 ± 71.6 ng mg⁻¹ albumin) compared with normal non-smokers (83.3 ± 2.9 ng mg⁻¹ albumin, P<0.01, 95% confidence interval was between 58.3 and 351.8).

Figure 2 (b) shows the concentrations of HGF in BALF in the study population. Data are expressed as means ± standard error. Hepatocyte growth factor in BALF in patients with IPF were significantly high (0.09 ± 0.03 ng mg⁻¹ albumin) compared with normal non-smokers (0.004 ± 0.007 ng mg⁻¹ albumin, P<0.01, 95% confidence interval was between 0.025 and 0.137). The ratio of HGF/albumin in BALF divided by serum HGF/serum albumin concentration was 12.3 ± 3.9.

Figure 3 (a) shows the correlation between plasma elastase:α1-proteinase complex values and serum HGF values in patients with IPF (n=31). Strong correlation was demonstrated (R=0.679, P<0.0001).

Figure 3 (b) shows the correlation between elastase:α1-proteinase complex values in BALF and HGF values in BALF in patients with IPF (n=15). No significant correlation was demonstrated (R=0.197, P=0.4883).
Plate 1 shows the results of immunostain by anti-human neutrophil elastase in IPF lung specimens. Immunopositive cells were observed mainly in the interstitium. Neutrophil elastase stains were detected around the fibrosis, as well as the alveolar septa. Some immunopositive cells were observed in the alveolar space. Strong staining of neutrophils was observed along the endothelium in pulmonary microvessels in some patients with IPF (figure not shown). There were few neutrophils distributed in the normal lung.

Plate 2 shows the results of immunostain by anti-HGF antibody. Immunostain by anti-HGF antibody was only possible in frozen sections and was impossible in paraffin sections. Immunohistochemical staining with rabbit polyclonal anti-human HGF antibody showed that HGF was distributed to the lung epithelial cells in IPF lung specimens. There was no significant staining to the alveolar epithelial cells in the normal lung (data not shown).

Discussion

Idiopathic pulmonary fibrosis is a disease of unknown aetiology that is characterized by the accumulation of neutrophils and mononuclear cells, followed by the progressive deposition of collagen within the interstitium and subsequent destruction of lung airspace. Significantly, patients with IPF have an expected median survival of less than 5 yr (5,6). Increases in polymorphonuclear neutrophils in BALF and in lung tissue have been demonstrated in patients with IPF (7). Excessive neutrophils in BALF have been associated with a higher likelihood of disease progression and a failure to respond to immunosuppressive therapy (8). Therefore, IPF has been considered to be a neutrophilic alveolitis since neutrophils may play an important role in the pathogenesis of IPF (4).

Neutrophil elastase, a neutral serine protease, is localized to the azurophilic or primary granules in neutrophils, and plays an important role in host defence (9). It has been reported that the formation of the plasma elastase:α1-proteinase complex reflects a rapid response of host to infection and is a sensitive indicator of bacterial infections during and beyond the neonatal period (10,11). In addition, sequential determinations of the elastase:α1-proteinase complex seem to be helpful in following the course of systemic bacterial infections and to determine the adequacy of therapy (11,12). In the present study, the increase of elastase:α1-proteinase complexes in plasma and BALF was demonstrated in patients with IPF.
In this study, neutrophil elastase was also used as a neutrophil marker in immunostain to distinguish neutrophils in the lung. It is especially useful in cases where many of the infiltrating neutrophils are premature (have a non-segmented nucleus). In this study, to evaluate the site of neutrophil accumulation in patients with IPF, specimens were obtained by open lung biopsy and were immunohistochemically stained using anti-human neutrophil elastase. Neutrophil elastase stains were detected around the fibrosis, as well as the alveolar septa. The authors also reported recently that strong staining of neutrophils was observed along the endothelium in pulmonary microvessels in some patients with IPF (13). These observations suggest that activation of neutrophils at the site of pulmonary interstitium takes place in patients with IPF. These results suggested that neutrophil elastase may play a role in the pathogenesis of IPF.

**Fig. 3.** Correlation between (a) plasma elastase-α1-proteinase PI complex values and serum hepatocyte growth factor (HGF) values, and (b) elastase-α1-proteinase complex values in bronchoalveolar lavage fluid (BALF) and HGF values in BALF, in patients with idiopathic pulmonary fibrosis.

**Plate 1.** Immunostain by anti-human neutrophil elastase in idiopathic pulmonary fibrosis lung specimens. Neutrophil elastase stains were detected around the honey-combing, as well as the alveolar septa. Some immunopositive cells were observed in the alveolar space.

**Plate 2.** Immunostain by anti-hepatocyte growth factor antibody. Hepatocyte growth factor was distributed to the lung epithelial cells in idiopathic pulmonary fibrosis lung specimens.

It has been reported that HGF is a growth factor that may mediate human renal cyst formation (14). As in renal cyst formation, the process of lung remodelling also includes epithelial cell proliferation and a remodelling of the extracellular matrix. In the lung, HGF is synthesized and secreted by mesenchymal cells, such as macrophages, endothelial cells and fibroblasts, and it controls proliferation and morphogenesis of epithelial cells (1). It has recently been revealed that the lung has an endocrine function and produces HGF for the regeneration of injured tissues or organs (2). Hepatocyte growth factor also acts as a pulmotrophic factor in lung regeneration after acute lung injury (3), and is a potential paracrine growth factor for rat alveolar type II cells in primary culture (1). In the present study, the increase of HGF in serum and BALF was demonstrated in patients with
IPF. Therefore, one may hypothesize that this HGF may play an important role in regulating the growth of the lung epithelium and in the regeneration of the lung in IPF.

The clinical significance of HGF in patients with IPF should be considered. Maeda et al. have suggested that serum HGF levels increase in inflammatory lung disease including interstitial pneumonitis, and HGF levels in the surviving patients rapidly decrease with treatment (15). The present authors also showed previously that plasma HGF concentration in patients with pneumonia was significantly higher when compared to patients without pneumonia, and the increase of serum HGF followed shortly after the onset of inflammation (16). This may suggest that HGF is an indicator of lung repair after lung damage in patients with pneumonia (16). Although the increase of HGF in serum may not be specific to IPF, future studies are needed to determine the clinical usefulness of measuring and monitoring serum HGF in patients with IPF.

Hepatocyte growth factor is thought to be produced by mesenchymal cells. However, immunohistochemical staining with anti-human HGF antibody showed that the HGF was distributed to the lung epithelial cells in the IPF lung. Defrances et al. also found that HGF is localized in the bronchial epithelium of developing rats, using an immunohistochemical study (17). They also showed that HGF is produced by mesenchymal cells in each organ, which might regulate epithelial cells growth and morphogenesis (17). Yanagita et al. reported that following intratracheal HCl injection, HGF mRNA and HGF activity in the lung increased, and the HGF receptor on the plasma membranes of the lung was downregulated, probably due to internalization of the HGF receptor following HGF binding (2,18). In the present study, the ratio of HGF/albumin in BALF divided by serum HGF/serum albumin concentration was 12.3 ± 3.9. Although the source of HGF in alveolus was not clear, this evidence suggested that HGF might be locally produced in the alveolus.

In conclusion, these results suggest that serum HGF increased in patients with IPF, and significantly correlated with the plasma elastase: α1-proteinase complex. Hepatocyte growth factor may play an important role in the pathogenesis of IPF.

References


