Raised plasma concentrations of α-defensins in patients with idiopathic pulmonary fibrosis

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Background: Neutrophils are thought to play an important role in the pathogenesis of idiopathic pulmonary fibrosis (IPF). Human neutrophils contain antimicrobial and cytotoxic peptides in the azurophil granules which belong to a family of mammalian neutrophil peptides named α-defensins. A study was undertaken to investigate the role of α-defensins in the pathogenesis of IPF.

Methods: The concentrations of α-defensins (human neutrophil peptides (HNPs) 1, 2, and 3) in plasma and bronchoalveolar lavage (BAL) fluid of 30 patients with IPF and 15 healthy subjects were measured by radiolimunoassay.

Results: The concentrations of α-defensins in plasma, but not in BAL fluid, were significantly higher in IPF patients than in controls. BAL fluid concentrations of interleukin (IL)-8 in patients with IPF, which were significantly higher than in controls, correlated with those of α-defensins. An inverse relationship was seen between plasma α-defensin levels and the arterial oxygen tension (PaO2) and pulmonary function (vital capacity (%VC), forced expiratory volume in 1 second (FEV1), and carbon monoxide transfer factor (%TLCO)) in patients with IPF. Plasma levels of α-defensins also correlated with the clinical course in IPF patients with an acute exacerbation. Immunohistochemically, positive staining was observed inside and outside neutrophils in the alveolar septa, especially in dense fibrotic areas.

Conclusion: These findings suggest that α-defensins play an important role in the pathogenesis of IPF, and that the plasma α-defensin level may be a useful marker of disease severity and activity.
Fifteen age and sex matched healthy volunteers were included as controls. All normal volunteers had normal chest radiographs, were free of symptoms, and were not taking any medication.

Bronchoalveolar lavage

With informed consent, BAL was performed as described previously using a flexible fiberoptic bronchoscope (Olympus, P-20 Olympus, Tokyo, Japan). Briefly, the bronchoscope was wedged into one of the segmental or subsegmental bronchi of the right middle lobe and 50 ml sterilised saline at body temperature was then instilled through the bronchoscope. The fluid was immediately retrieved by gentle suction using a flexible fibreoptic bronchoscope (Olympus, Tokyo, Japan). A diluted sample or standard peptide solution (100 µl) was incubated for 24 hours with 100 µl antiserum diluent by RP-HPLC on a TSK ODS 120A column (Toso Co, Tokyo, Japan). A diluted sample or standard peptide solution (100 µl) was incubated for 24 hours with 100 µl antiserum diluent (final dilution 1/21 000). The I-labelled HNP-1 solution (16 000 cpm in 100 µl) was added and the mixture was incubated again for another 24 hours. Normal rabbit serum and anti-rabbit IgG goat serum were then added and stored for 16 hours. Bound and free ligands were separated by centrifugation. All procedures were performed at 4°C and duplicate assays were carried out. Volumes of 0.5 µl plasma and 1–10 ml BAL fluid were used to determine the levels of α-defensins. The antiserum recognised HNP-1, HNP-2, and HNP-3 equally on a molar basis, so the RIA data were expressed as the sum of HNPs 1–3 and their precursor proteins, the presence of which was confirmed by simultaneous measurements using RP-HPLC and RIA. The intra-assay and inter-assay coefficients of variation were 3.5% and 8% at 50% binding, respectively, in this RIA.

Serum samples were used to measure pulmonary surfactant protein (SP)-A, SP-D, and KL-6 which are known markers of disease activity in interstitial lung diseases. SP-A (the Teijin Institute of Bio-medicine, Tokyo, Japan), SP-D (SP-D kit Yamasa, Yamasa Shoyu Co, Tokyo, Japan), and KL-6 (ED 406, Eisai, Tokyo, Japan) assays were performed using commercially available ELISA kits. The concentration of interleukin (IL)-8 was also measured with a commercially available kit (Toray Fuji Bionics, Tokyo, Japan).

Immunohistochemistry of lung tissues

Immunohistochemical analysis of α-defensins in the lungs of patients with IPF was performed as described previously. Surgical lung biopsy specimens were obtained from six IPF patients. As a control, normal lung tissue specimens resected at surgery from a 45 year old man with tuberculosis were also studied. The tissue samples were immersed in Zamboni’s fixative (2% paraformaldehyde and 0.25% picric acid in 0.1 M PBS) or in 10% formaldehyde in PBS and, after dehydration in serial ethanol concentrations, they were embedded in paraffin. The specimens (3 mm thick) were deparaffinised in xylene, rehydrated in serial ethanol solutions, and treated with 0.3% hydrogen peroxide for 30 minutes.
to inactivate any endogenous peroxidase. Non-specific binding was blocked with normal goat serum. Anti-HNP 1–3 antiserum at a final dilution of 1/10 000 was allowed to react overnight with each preparation at 4°C in a moist chamber. Goat biotinylated anti-rabbit IgG was used as the second antibody. The samples were stained by the ABC alkaline phosphatase (ABC-AP) method using an ABC-AP kit (Dako Co, Carpinteria, CA, USA) and the tissue samples were counterstained with haematoxylin.

**Statistical analysis**

Data were expressed as mean (SD) values. Differences between groups were examined using the Mann-Whitney U test. Correlations between two groups were determined using the Spearman’s rank correlation analysis. A p value of <0.05 was considered to be statistically significant.

**RESULTS**

**BAL fluid findings and α-defensin levels**

The total number of cells recovered in the BAL fluid was significantly higher in patients with IPF than in controls (table 1). In patients with IPF the percentage of alveolar macrophages was lower and the number and percentage of neutrophils were significantly higher than in controls (table 1). There were no significant correlations between the number and percentage of neutrophils in BAL fluid and various clinical parameters including arterial oxygen tension (PaO₂) and pulmonary function tests. As shown in fig 1, the mean plasma concentration of α-defensins was higher in patients with IPF (768.2 (422.4) ng/ml) than in controls (323.3 (173.1) ng/ml, p<0.01), but there was no significant difference between the two groups in the concentration of α-defensins in the BAL fluid (IPF: 52.0 (92.0) ng/ml; control: 12.9 (15) ng/ml).

There was no significant correlation between plasma and BAL fluid concentrations of α-defensins in patients with IPF, but the number (r=0.395, p=0.034) and percentage (r=0.436, p=0.019) of neutrophils correlated with the concentrations of α-defensins in the BAL fluid of IPF patients. Plasma concentrations but not BAL fluid concentrations of α-defensins correlated negatively with PaO₂, %VC, FEV₁, and %TLCO in patients with IPF (fig 2 and table 2). A correlation analysis of the levels of KL-6, SP-A, and SP-D (which are known markers of disease activity in interstitial lung diseases) with these clinical parameters revealed only significant correlations between SP-A and %VC or %TLCO (table 2). Plasma α-defensin concentrations in patients with IPF did not correlate with the serum concentrations of KL-6 (r=−0.223, p=0.346), SP-A (r=−0.273, p=0.26), or SP-D (r=−0.297, p=0.236).

As shown in fig 3, concentrations of IL-8 in both BAL fluid (37.2 (64.2) pg/ml) and serum (27.5 (69.8) pg/ml) in patients with IPF were significantly higher than in control subjects (5.9 (5.7) pg/ml in BAL fluid; 0.9 (2.4) pg/ml in serum). Although no significant correlation was observed between the BAL fluid concentration of IL-8 and the plasma concentration of α-defensins, there was a positive correlation between α-defensins and IL-8 in BAL fluid (r=0.533, p=0.0041, fig 4). A significant correlation was also observed between IL-8 levels and the number (r=0.424, p=0.0217) and percentage (r=0.491, p=0.0079) of neutrophils in the BAL fluid of patients with IPF.
The clinical course and serial changes in plasma concentrations of α-defensins in two representative IPF patients with acute exacerbations are depicted in Fig 5. In one patient an acute exacerbation was associated with a rise in the plasma concentration of α-defensins while clinical improvement and resolution of chest radiographic findings induced by steroid and cyclophosphamide treatment were associated with a fall in the plasma α-defensin concentration (Fig 5A). Two patients with IPF died of respiratory failure due to an acute exacerbation with a concomitant increase in plasma concentrations of α-defensins. The clinical and laboratory data of one of these two patients are shown in Fig 5B.

**DISCUSSION**

We have evaluated the role of neutrophils in patients with IPF through its granule protein, α-defensins. The major finding was that patients with IPF had significantly increased concentrations of α-defensins in plasma but not in BAL fluid. In addition, the plasma levels of α-defensins correlated inversely with several clinical parameters including PaO₂ and pulmonary function tests (%VC, FEV₁, and %TLCO) in patients with IPF. Immunohistochemical studies also demonstrated the accumulation of a considerable number of neutrophils in lung parenchyma biopsy specimens.

Increased numbers of neutrophils in BAL fluid and lung tissue have been reported in patients with IPF. However, the neutrophil count in the BAL fluid has not been shown to have a prognostic value. We found no correlation between the number of neutrophils and the BAL fluid concentration of α-defensins or clinical parameters, although there was a significantly higher number of neutrophils in the BAL fluid of patients with IPF. Obayashi and colleagues' found increased numbers of infiltrating neutrophils in the lung parenchyma of IPF patients using immunohistochemistry for neutrophil elastase, but no increase in neutrophil count in the BAL fluid.
α-Defensins in IPF

These findings suggest that the BAL procedure does not sample the underlying interstitial process accurately in patients with IPF. Our study showed an inverse relationship between plasma concentrations of α-defensins and PaO₂, %VC, FEV₁, and %Tlco in patients with IPF. In this context, Yamanouchi et al. found increased serum levels of E-PI, another neutrophil granule protein, in patients with IPF, and a positive correlation between serum and BAL fluid levels of E-PI and some clinical parameters including PaO₂. The present immunohistochemical studies (for α-defensins) and those of other laboratories for neutrophil elastase showed that positive staining was observed both inside and outside neutrophils in the alveolar sepa, especially in dense fibrotic areas. These findings suggest the involvement of neutrophils through their cytotoxic granules in the pathogenesis of pulmonary fibrosis. This is supported by a previous report which showed that administration of neutrophil elastase inhibitor attenuated the severity of bleomycin induced lung injury and of subsequent pulmonary fibrosis. The plasma concentration of α-defensins, as well as neutrophil elastase, may therefore be a useful marker of disease severity in IPF. In addition, our finding that plasma concentrations of α-defensins reflect the clinical course in patients with an acute exacerbation suggests that the plasma concentration of α-defensins is also a useful marker of disease activity.

Using immunohistochemistry, we also found the expression of α-defensins in neutrophils attached to the capillary endothelium (fig 5C). This finding is similar to that reported by Obayashi et al and suggests that activation of neutrophils at the site of the endothelium of pulmonary microvessels may take place in patients with IPF. Westlin and colleagues have shown the direct cytotoxic effect of neutrophils on human vascular endothelium, and α-defensins also have a direct cytotoxic effect on human endothelial cells. Neutrophil endothelitis and part of “neutrophilic alveolitis” could therefore be an important pathogenic feature of IPF.

Our finding of a positive correlation between IL-8 and α-defensin levels in the BAL fluid of patients with IPF fits well with previous reports of the relationship between IL-8 and α-defensins in various types of pulmonary infectious diseases. IL-8 is a potent neutrophil attractant that can induce the release of α-defensins from neutrophils, which subsequently stimulate IL-8 synthesis by airway epithelial cells. The relationship between IL-8 and IPF is now well known, and alveolar macrophages located predominantly within the alveolar spaces are the major source of IL-8 in IPF. Thus, IL-8 may be a key cytokine in the accumulation of neutrophils and the high concentration of α-defensins in BAL fluid in this disease. However, the concentrations of both IL-8 and α-defensin in the BAL fluid of patients with IPF were lower than in patients with DPB (α-defensins: 429 ng/ml, IL-8: 324 pg/ml) and Mycobacterium avium-intracellulare infection (α-defensins: 680 ng/ml, IL-8: 568 pg/ml). This finding may merely reflect the smaller number of neutrophils in the BAL fluid of patients with IPF (0.2 (0.3) × 10⁷/ml) compared with DPB (9.8 (2.4) × 10⁷/ml) and M avium-intracellulare infection (3.5 (1.8) × 10⁷/ml).

We have previously shown that BAL fluid levels of granulocyte colony-stimulating factor (G-CSF) in patients with IPF, which were higher than in other lung diseases, correlated with the BAL fluid neutrophil count, and that in BAL fluid samples from patients with IPF the mean value of neutrophil chemo-attractant activity was reduced by 35% after neutralisation with anti-human G-CSF antiserum. Administration of recombinant human G-CSF in patients with lung cancer enhances α-defensin biosynthesis in neutrophils. Thus, G-CSF might also be involved in neutrophil alveolitis and high plasma concentrations of α-defensins in patients with IPF. SP-A, SP-D, and KL-6 are useful biomarkers of interstitial lung diseases. SP-A and SP-D belong to the collectin subgroup of the C-type lectin superfamily. They are produced by two types of epithelial cells in the peripheral airway—Clara cells and alveolar type II cells—and their levels can predict IPF disease activity. The serum level of KL-6, MUC1 mucin, derived from damaged or regenerating type II pneumocytes in IPF is also useful for the diagnosis and evaluation of the activity of the disease. In our study we could not find any correlation between plasma concentrations of α-defensins and serum concentrations of SP-A, SP-D, or KL-6, probably because of differences in the origin of these markers. In addition, the plasma level of α-defensins was the most useful marker for evaluating disease severity in this study (table 2). Although...
the plasma concentration of α-defensins is not necessarily a useful marker for the clinical diagnosis of IPF as raised levels are also seen in other lung diseases. It is a distinct new marker for disease severity and activity in IPF.

In conclusion, we have shown that plasma concentrations of α-defensins were raised in patients with IPF. Our findings suggest that neutrophil related pulmonary dysfunction may be mediated through α-defensins in IPF, and that these peptides could serve as new parameters of disease severity and activity.

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