Gastrin-releasing peptide-like immunoreactive substance in bronchoalveolar lavage of idiopathic pulmonary fibrosis and sarcoidosis


ABSTRACT: The neuropeptide gastrin releasing peptide (GRP) is present in the lung, and functions as a modulator of tissue growth and repair in fibrotic processes, or as a modulator of cell movement and differentiation in various inflammatory processes, including granulomatous ones. In idiopathic pulmonary fibrosis (IPF), changes in the bronchoalveolar lavage (BAL) content of GRP can be expected.

We measured GRP-like immunoreactive substances (GRP-IS) and another neuropeptide, vasoactive intestinal peptide (VIP-IS) in BAL by enzyme immunoassay.

Our results showed a decrease in BAL GRP-IS in patients with IPF (26.5±5.5 pg·mg⁻¹ protein) and sarcoidosis (35.9±9.2 pg·mg⁻¹), compared to healthy nonsmokers (63.4±9.0 pg·mg⁻¹). When data were expressed as pg·ml⁻¹ BAL fluid recovered, a decrease was only seen in IPF, not in sarcoidosis.

The levels of VIP-IS in BAL were not different between the groups studied. Increased protein levels in BAL had no correlation with the levels of GRP-IS or VIP-IS in BAL. Furthermore, BAL neutrophil percentages had no correlation with the levels of GRP-IS in BAL of patients with IPF. Using reversed phase high performance liquid chromatography (HPLC), several kinds of GRP-IS were detected in BAL.

These findings suggest that the decreased level of GRP-IS in BAL may reflect a loss of GRP-producing cells due to chronic lung injury and fibrosis in patients with IPF.

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Human gastrin-releasing peptide (GRP) was isolated, and its amino acid sequence was determined in 1984 [1, 2]. This 27 amino acid residue peptide is widely distributed in mammalian tissues, and has various biological effects in mammals [3]. GRP is present in the neuroepithelial bodies [4-6], nerve endings [7] and macrophages [8, 9] of the lung. GRP containing neuroepithelial bodies are common in the developing lung, and decline with increasing age; and GRP is found to be a growth factor for normal human bronchial epithelial cells [10], small cell carcinoma [11, 12], and fibroblasts [13]. Therefore, GRP may play an important role in the regulation of tissue growth and repair in the lung. It has also been reported that chronic smoking or chronic hypoxia causes an increase in lavage fluid GRP levels [14, 15]. Besides GRP, vasoactive intestinal peptide (VIP), which was first isolated from porcine small intestine, has been demonstrated to be localized in neurons and nerve fibres in the adult lung [16].

In patients with idiopathic pulmonary fibrosis (IPF), the fibrotic process can be regarded as an exaggerated repair of lung injury. It has been reported that macrophages play an important role in both lung injury and fibrosis in patients with IPF [17]. Recently, interest in other lung cells, including epithelial cells, has arisen, with respect to inflammatory processes and induction of fibrosis [18].

In patients with pulmonary sarcoidosis, the activation/differentiation of monocytes, which are accumulated in the lung due to monocyte chemotactic factors, is considered to be strongly connected with the formation of epithelioid cell granuloma. It has been reported that GRP is a chemotaxant to monocytes [19].

In this study, we examined the level of GRP and VIP in bronchoalveolar lavage (BAL) fluids in patients with IPF and pulmonary sarcoidosis.

Materials and methods

Study population

We studied 14 cases of IPF, 20 cases of pulmonary sarcoidosis, 14 healthy nonsmokers and 19 healthy smok-
ers. All cases of IPF were diagnosed by open lung biopsy, which showed the histological appearance of usual interstitial pneumonia. All studied cases with IPF showed restrictive pulmonary dysfunction (vital capacity (VC) 72±17%, diffusion capacity of the lung for carbon dioxide (Dcco) 47±12%) and mild hypoxemia, arterial oxygen tension (Pao₂) 74.3±11.4 mmHg (9.9±1.5 kPa), arterial carbon dioxide tension (Paco₂) 41.3±5.4 mmHg (5.5±0.7 kPa). The diagnosis of pulmonary sarcoidosis was made by transbronchial lung biopsy. Twelve cases showed bilateral hilar lymphadenopathy (BHL), two cases showed parenchymal lesion, and six cases showed both abnormalities on chest radiograph.

All sarcoidosis cases showed normal pulmonary function. None of the subjects had any signs or symptoms of infections. None of the studied cases received corticosteroid therapy. The lavage study of human subjects received approval from the Ethics Committee of Chest Disease Research Institute Hospital, Kyoto University.

**Bronchoalveolar lavage**

BAL was performed as described previously with a slight modification [20]. Briefly, the upper airways were anaesthetized with 4% lidocaine (Xylocaine). The fibreoptic bronchoscope was inserted into the tracheobronchial tree and wedged in the right middle lobe. A lavage was performed using warm (37°C) saline solution (6x50 ml). Recovered BAL fluid was filtered through gauze and centrifuged at 200xg for 10 min at 4°C. The cell pellet was resuspended in Eagles minimal essential medium (MEM) and centrifuged again to adjust the total cell count. A differential cell count was performed from cytopsin smears after May-Grünewald-Giemsa staining. The cell free supernatants were immediately frozen at -20°C until use. The protease inhibitor aprotinin (250 KIU·ml⁻¹) was added to the supernatants to prevent peptides from being cleaved by proteolytic enzymes in the supernatants.

The determination of protein concentration in BAL was performed according to the method of Lowry et al. [21].

**Antibodies**

Antiserum to human GRP (GP6201), was prepared as described previously by Takeyama et al. [22]. Briefly, synthetic human GRP and bovine serum albumin (BSA) were conjugated by the water soluble carbodiimide coupling method, and the conjugate with Freund's complete adjuvant was administered to Japanese white rabbits. Antiserum GP6201 with a high titre was diluted with the assay buffer (PH 7.2, 0.05 M phosphate buffer, supplemented with 0.5% BSA, 1 mM MgCl₂ and aprotinin 250 KIU·ml⁻¹) to 1:40,000 and used for enzyme immunoassay (EIA) of human GRP. Anti-VIP antibody (604/001) was purchased from UCB-Bioproducts (Belgium).

**Extraction of GRP-IS and VIP-IS from BAL fluid**

The extraction of GRP-IS and VIP-IS from BAL fluid was performed as reported previously [23]. Briefly, each BAL fluid sample (15 ml) was loaded on a reversed-phase C18 cartridge (SEP-PAK, Waters Associate Inc., Milford, USA). After washing with 10 ml of 4% acetic acid (AcOH, pH 4.0), GRP-IS and VIP-IS were eluted with 2 ml of 70% acetonitrile in 0.5% AcOH. Eluates were concentrated by spin-vacuum evaporation, and lyophilized. The lyophilized extracts were dissolved in 100 μl assay buffer and subjected to enzyme immunoassay. The extraction efficiency was assessed by recovery experiments. When synthetic GRP was added to BAL samples (15 ml) at a final concentration of 5 and 20 pg·ml⁻¹, the recovery of GRP was 96±6%. Similarly, the recovery of VIP with the extracting procedure was 95±4%.

**Enzyme immunoassay (EIA)**

EIA for GRP was performed as described previously [22]. Carboxy terminal (C-terminal) human GRP fragment, GRP (12-27) was labelled with β-D-galactosidase. EIA was performed by the delayed addition method. The separation between free and bound form was performed by the double antibody solid phase method. The detectable minimum amount of GRP by this method was 6.0 fmol·ml⁻¹. EIA for VIP was done in essentially the same way, except the antiserum for VIP (604/001) detected the central region of human VIP (hVIP) and the sensitivity was 1.5 fmol·ml⁻¹ [24].

**HPLC of BAL fluid extracts**

For biochemical characterization of GRP-IS, BAL fluid extracts were applied to reversed phase HPLC. HPLC was performed using a reversed phase C18 column (Wakosil 5C18, 150×4.6 mm 1D, 10 μM, Wako Pure Chemical Industries, Ltd, Osaka, Japan). The column was equilibrated with 0.1% trifluoroacetic acid (TFA). BAL fluid extracts (40 ml from healthy nonsmokers, smokers, patients with IPF and sarcoidosis) were applied to the column. After washing with 0.1% TFA at a flow rate of 1 ml·min⁻¹ for 5 min, GRP-IS was eluted by two steps of linear gradients of acetonitrile (0-20% in 5 min and 20-50% in 45 min) in 0.1% TFA. Each fraction (1 ml) was concentrated by spin-vacuum evaporation, and lyophilized. The residue was reconstituted to 100 μl with assay buffer and submitted to EIA. Synthetic human GRP and its fragments were applied to the HPLC under the same conditions, and their retention times were determined.

**Statistical analysis**

All results were expressed as mean±sd. The significance of differences between the studied groups was evaluated by using the one-way analysis of variance. A value of p<0.05 was considered to be significant.
Results

Basic BAL findings

Basic BAL findings are summarized in Table 1. Significant decreases in fluid recovery, and macrophage percentage, and significant increases in neutrophil percentage, eosinophil percentage and protein concentration were found in patients with IPF, compared to healthy nonsmokers.

In patients with sarcoidosis, significant increases in lymphocyte percentage, CD4+/CD8+ ratio, and protein concentration were detected, compared to healthy nonsmokers. Macrophage percentage was decreased.

Table 1. - Basic BAL findings

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Smokers</th>
<th>IPF</th>
<th>Sarcoidosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases n</td>
<td>14</td>
<td>19</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>Age yrs</td>
<td>40±2</td>
<td>43±3</td>
<td>61±3*</td>
<td>40±4</td>
</tr>
<tr>
<td>BAL fluid:</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fluid recovered %</td>
<td>75±6.7</td>
<td>69±11.3</td>
<td>60±16.6*</td>
<td>72±8.6</td>
</tr>
<tr>
<td>Recovered cells x10^6·ml⁻¹</td>
<td>1.2±1.5</td>
<td>3.4±5.4</td>
<td>2.1±0.9</td>
<td>6.9±11.3</td>
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<td>Macrophage %</td>
<td>95.2±3.8</td>
<td>95.7±7.4</td>
<td>90.3±7.0**</td>
<td>77.4±18.9*</td>
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<tr>
<td>Lymphocytes %</td>
<td>4.6±3.7</td>
<td>3.3±5.0</td>
<td>4.6±3.5</td>
<td>20.8±18.4*</td>
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<tr>
<td>Eosinophils %</td>
<td>0.2±0.3</td>
<td>0.7±2.3</td>
<td>2.8±3.0*</td>
<td>1.4±3.7</td>
</tr>
<tr>
<td>Neutrophils %</td>
<td>0.0±0.1</td>
<td>0.3±0.9</td>
<td>2.3±2.5*</td>
<td>0.4±0.8</td>
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<tr>
<td>CD4/CD8 ratio</td>
<td>2.4±0.8</td>
<td>1.2±1.1*</td>
<td>1.6±1.1</td>
<td>5.3±3.2*</td>
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<tr>
<td>Total protein mg·ml⁻¹</td>
<td>0.08±0.03</td>
<td>0.09±0.04</td>
<td>0.15±0.08*</td>
<td>0.25±0.41</td>
</tr>
</tbody>
</table>

Values are expressed as mean±sd. Significant difference *: p<0.01, **: p<0.05, compared with healthy nonsmokers. BAL: bronchoalveolar lavage; IPF: idiopathic pulmonary fibrosis.

Immunospecificity of the GRP-antisem (GP6201) was examined by EIA using β-D-galactosidase labelled GRP (12-27). As shown in Figure 1, the carboxyterminal (C-terminal) GRP fragment, GRP (18-27) exhibited 100% cross-reactivity as compared to GRP, while bombesin had less than 1%. Neumedin B, litorin and ranatensin hardly exhibited cross-reactivity (data not shown). Thus, it was shown that our GRP antisem could precisely recognize the C-terminal region of GRP and distinguish GRP from bombesin and other GRP-related peptides.

As also shown in Figure 1, our GRP-EIA was highly sensitive and allowed detection of 6-600 fmol·ml⁻¹ of GRP, which was fifty fold higher than that of bombesin-EIA (0.3-30 pmol·ml⁻¹) reported by AGUAYO et al. [14].

GRP-IS and VIP-IS in BAL

The displacement curves parallel to the standard curve for synthetic GRP were obtained with extracts of BAL fluid (Figure 1). Similar results were obtained with VIP (data not shown).

The BAL samples used to determine GRP-IS and VIP-IS in this study were analysed according to the same procedure, using human milk, as in the previous report [22]. The levels of GRP-IS and VIP-IS in BAL fluid of healthy nonsmokers, smokers, patients with IPF and sarcoidosis are shown in Figure 2. The mean levels of GRP-IS in BAL fluid of healthy nonsmokers and smokers were 4.7±0.7 and 4.0±0.8 pg·ml⁻¹, respectively.

In patients with IPF, the level of GRP-IS was 2.9±0.5 pg·ml⁻¹ or 26.5±5.5 pg·mg⁻¹ protein. This level was significantly lower, compared to that of healthy nonsmokers, whether expressed as pg·ml⁻¹ (p<0.05) or pg·mg⁻¹ protein (p<0.01). Also, the level of GRP-IS in patients with IPF did not correlate with BAL fluid
DECREASE OF GRP IN BALF OF IPF AND SARCOIDOSIS

In patients with sarcoidosis, the level of GRP-IS (4.7±0.9 pg·mL⁻¹) was not different from that of healthy nonsmokers, but when the level was expressed as pg·mg⁻¹ protein (35.9±9.2 pg·mg⁻¹ protein), it was lower in comparison with that of healthy nonsmokers (p<0.05). The decrease was not correlated with recovered cells, lymphocytes, macrophages and protein concentration.

The levels of VIP-IS in BAL fluid were not different between all groups studied, whether expressed as pg·mL⁻¹ or pg·mg⁻¹ protein (fig. 2).

During storage of BAL fluid samples, whether the protease inhibitor aprotinin was added or not, the levels of peptides were not different (data not shown).

Furthermore, levels of GRP-IS were almost the same before and after 6 months storage of BAL fluid samples (data not shown).

HPLC of BAL fluid extracts

For biochemical analysis, BAL fluid extracts were subjected to HPLC by using a reversed phase C₁₈ column. The elution profiles of the BAL fluid extracts from healthy nonsmokers, smokers, patients with IPF and sarcoidosis are shown in figure 3. Several kinds of GRP-IS were observed in all BAL fluid extracts. In each subject, one of the GRP-IS was identified with the synthetic human GRP at the region of 36% acetonitrile, and the other was identified with the synthetic human GRP (18-27) at the region of 31% acetonitrile.

Fig. 2. – Levels of GRP-IS and VIP-IS in BAL fluid of healthy nonsmokers (NS), healthy smokers (SM), patients with idiopathic pulmonary fibrosis (IPF) and sarcoidosis. The levels of GRP-IS and VIP-IS are shown as pg·mL⁻¹ (left) and pg·mg⁻¹ protein (right). Horizontal bars represent mean values. A) Levels of GRP-IS: NS (n=14), SM (n=19), IPF (n=14), and Sar (n=20). B) Level of VIP-IS: NS (n=9), SM (n=13), IPF (n=12), and Sar (n=8). GRP-IS: gastrin releasing peptide-like immunoreactive substances; VIP-IS: vasoactive intestinal peptide-like immunoreactive substances.
Discussion

In this study, we demonstrated that lower levels of BAL fluid GRP-IS were detected in patients with IPF. Conversely, there was no decrease in levels of VIP-IS in these patients. It is less likely that GRP-IS is severely cleaved by proteolytic enzymes in BAL fluid of patients with IPF or sarcoidosis, because the activities of their proteolytic enzymes were similar to those of healthy nonsmokers (data not shown).

Taken together, there is a possibility of loss of GRP-producing cells due to chronic lung injury and fibrosis in patients with IPF. Since the levels of VIP-IS in BAL fluid from patients with IPF and sarcoidosis were not different from those in healthy nonsmokers, it remains to be elucidated whether the two neuropeptides are localized in a similar distribution in the lungs.

In patients with sarcoidosis, no decrease in GRP-IS was detected when data were expressed as pg·ml⁻¹ of BAL fluid. A significant decrease was shown only when expressed as pg·mg⁻¹ protein of BAL fluid. The amount of protein in BAL fluid of sarcoidosis was increased compared to that in healthy nonsmokers. Therefore, the decrease in GRP-IS, when expressed in pg·mg⁻¹ protein, is due to an increase in protein in BAL fluid from patients with sarcoidosis, probably caused by increased capillary permeability, and does not reflect a true decrease in the alveolar lining fluid.

Regarding the specificity of our assay, our anti-GRP serum was specific to the carboxyterminal of human GRP (hGRP) which belongs to the bombesin family [22]. In addition, we confirmed the presence of GRP or GRP(18-27) in BAL fluid by using HPLC.

It has been reported, in agreement with our results, that bombesin-IS level in BAL fluid from patients with IPF (13.8 pmol·ml⁻¹=22.3 ng·ml⁻¹) was decreased [26] compared to the level in healthy nonsmokers (26.6 pmol·ml⁻¹=43 ng·ml⁻¹) reported by the same group in a previous paper [14]. However, in contrast with our results, these authors reported that the level of bombesin-IS in BAL fluid from cigarette smokers was elevated [14]. Furthermore, their reported GRP-IS in BAL fluid (healthy nonsmokers: 26.6 pmol·ml⁻¹=43 ng·ml⁻¹, healthy smokers: 98.7 pmol·ml⁻¹=160 ng·ml⁻¹) was much higher than the GRP-IS level in our study (healthy nonsmokers: 1.6 fmol·ml⁻¹=4.7 pg·ml⁻¹, healthy smokers: 1.4 fmol·ml⁻¹=4.0 pg·ml⁻¹). This may be due to the difference in the EIA and GRP extraction method from BAL fluid. By the SEP-PAK extraction method used in our analysis, unknown substances disturbing the EIA could be removed. Unlike our anti-GRP serum, the anti-bombesin antibody (MoAb BBC353) detected bombesin, GRP, and their C-terminal fragments, ranatensin, litorin and neuromedin B [14]. Thus, their assay probably measured not only GRP but also many related peptides, explaining why their bombesin levels were higher than the GRP-IS levels in our results. Moreover, their bombesin-IS levels in BAL fluid were also much higher than that in developing lungs (5.2 pmol·g⁻¹ wet weight) as previously reported by Ghatei et al. [26]. Thus, the increased levels of bombesin-IS in BAL fluid from cigarette smokers reported by Aguayo et al. [25] might not
be caused by GRP but by other bombesin-related peptides. In lungs, GRP is thought to be localized in single neuroepithelial cells and neuroepithelial bodies, throughout the lungs, predominantly in the airways and nerve fibres around blood vessels and seromucous glands [4–6]. Neuroepithelial cells and neuroepithelial bodies, which are partially localized in the pulmonary epithelium, are considered as the predominant source of GRP in lungs [4–6].

There is a possibility that a decreased level of GRP-IS in BAL fluid reflects a chronic low grade lung injury in patients with IPF. This possibility is supported by an ultrastructural study showing alveolar epithelial cell injury in pulmonary fibrosis [27]. Alveolar epithelial cells are thought to contribute to the regulation of fibroblast proliferation in lungs [18].

Considering alveolar macrophages as one source of GRP-IS, it can be expected that GRP-IS plays an important role in producing epithelial cell granuloma in patients with sarcoidosis. Bombesin has been found to be a chemotaxator for monocytes [19], and also plays a significant role in cell differentiation [28]. Furthermore, immunofluorescence studies have indicated a heterogeneous distribution of bombesin-like immunoreactivity among alveolar macrophages [9]. This finding suggests that subpopulations of alveolar macrophages may contain a selective bombesin-like immunoreactivity. In animal models, an increased release of bombesin-like peptides by alveolar macrophages occurred only after the release of interleukin-1 [9]. Furthermore, the bombesin was not detected in fresh monocytes [8]. Therefore, the bombesin content may be related to the stage of alveolar macrophage differentiation and maturation. Whether lavage macrophages contain GRP-IS is unknown at present.

In conclusion, this study demonstrated a decrease in GRP-IS levels in BAL fluid of IPF patients. This may indicate a loss of GRP-producing cells due to the lung injury and fibrotic process in IPF.

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References


