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Lungs of patients with idiopathic pulmonary alveolar proteinosis express a factor which neutralizes granulocyte-macrophage colony stimulating factor

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Abstract Mice deficient in granulocyte-macrophage colony stimulating factor (GM-CSF) develop pulmonary alveolar proteinosis (PAP). We found that bronchoalveolar lavage fluid (BALF) from 11 patients with idiopathic pulmonary alveolar proteinosis (IPAP) suppressed the growth of peripheral blood monocytes and TF-1 cells, a cell line dependent on either GM-CSF or interleukin-3 (IL-3). The inhibitory effect of PAP-BALF occurred only when TF-1 cells were cultured with GM-CSF but not when cultured with IL-3, suggesting that PAP-BALF contains a factor that specifically interferes with GM-CSF function. ^{125}I-GM-CSF binding to TF-1 cells was prevented in the presence of BALF from IPAP patients. Furthermore, cross-linking of ^{125}I-GM-CSF to IPAP-BALF produced two major bands on SDS-PAGE; these bands were not observed in normal BALF. These data suggest that IPAP is caused by expression of binding factor(s) which inhibit GM-CSF function in the lung.

Key words: Alveolar macrophage; Peripheral blood monocyte; Bronchoalveolar lavage; Interleukin-3; Surfactant protein

1. Introduction

Pulmonary alveolar proteinosis (PAP) is a rare disease that causes progressive dyspnea. It is associated with intermittent accumulation of excessive surfactant composed of phospholipids and surfactant proteins designated SP-A, B, C, and D in the alveoli and terminal bronchioli [1,2]. When these materials are deposited in the alveoli, they block oxygen transport in the lung and sometimes cause severe hypoxemia. Three forms of PAP are known. The most common form is primary or idiopathic. A secondary form is associated with other diseases such as pulmonary infection, hematological malignancies, and inhalation of chemicals and minerals [3]. A congenital form is associated with hereditary SP-B deficiency [4] or defective expression of the common β chain of the granulocyte-macrophage colony stimulating factor (GM-CSF) receptor [5]. The etiology of idiopathic PAP (IPAP) is unknown. Recent clinical studies suggest that the disease is due to reduced clearance rather than excessive secretion of surfactant [6]. Because the bronchoalveolar lavage fluid (BALF) from PAP patients is enriched in surfactant protein A, the clearance of SP-A may be critically defective [7]. SP-A clearance is performed by alveolar macrophages (AM) and type II pneumocytes [8], which led Claypool and colleagues to suggest that AM in PAP are defective in surfactant processing or clearance [9]. Because IPAP is effectively treated by massive lavage [10], it is possible that lavage removes substance(s) from the lung that interfere with AM function.

Mice deficient in the gene for GM-CSF [11] or β chain of GM-CSF receptor [12,13] develop a PAP-like disease with alveolar accumulations of surfactant. These data demonstrate that GM-CSF signaling is necessary for surfactant homeostasis in the lung. The mouse model, therefore, raises the possibility that GM-CSF is involved in the pathogenesis of human PAP.

In this study we found that BALF from IPAP patients strongly inhibits the growth of monocytes and TF-1 cells, a GM-CSF/interleukin-3 (IL-3)-dependent cell line. The inhibition was specific to GM-CSF since TF-1 cells continue to grow in the presence of IL-3. We observed a factor in BALF from IPAP patients that binds to and neutralizes GM-CSF. The role of the factor in the pathogenesis of IPAP is discussed.

2. Materials and methods

2.1. Subjects

Eleven patients with IPAP, two patients with secondary PAP, and 10 control subjects underwent bronchoalveolar lavage. The diagnosis of PAP was confirmed by biochemical analysis of BALF and histopathological findings of lung biopsy. IPAP patients demonstrated no underlying disease including hematological disorders, infectious diseases, or toxic inhalation. The two patients with secondary PAP had chronic myelogenous leukemia. Written informed consent to participate in the study was obtained from all subjects.

2.2. Bronchoalveolar lavage fluid

BALF was centrifuged at $1000\times g$ for 15 min. The supernatant was then centrifuged at $40\,000\times g$ for 60 min. The supernatant was defined as 'the BALF'. Protein concentration of the BALF was measured by the method of Bradford [14] using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction.

2.3. Cytokines and antibodies

Recombinant human (rh) GM-CSF (specific activity 2.25×10^8 U/mg) was kindly provided by Kirin Brewery Co. Ltd. (Takasaki, Japan), rhIL-3 was purchased from R&D (Minneapolis, MN, USA). Rat anti-hGM-CSF monoclonal antibody 23B6 [15] was kindly provided by Dr. T. Kitamura (The Institute of Medical Science, The University of Tokyo, Tokyo, Japan). Peroxidase labeled anti-hGM-CSF polyclonal antibody was purchased from R&D Systems and ^{125}I-Bolton Hunter labeled rhGM-CSF from NEN Life Science Products (Boston, MA, USA).

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2.4. Preparation of human monocytes and TF-1 cells

Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors by Ficoll/Paque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation, resuspended in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% heat inactivated fetal calf serum (FCS). PBMC were incubated for 15 min at 37°C in 250 ml tissue culture flasks (Falcon, Franklin Lakes, NJ) coated with 10% human AB serum in PBS. Non-adherent cells were removed by vigorous washing with PBS and adherent cells were collected by gentle scraping using a cell scraper (Sumitomo Bakelite, Tokyo, Japan) after incubation with 0.05% trypsin/0.53 mM EDTA for 10 min at 37°C. At least 98% of the adherent cells were monocytes by morphology and non-specific esterase staining. The viability of cells was determined by the trypan blue dye exclusion. TF-1 is a cell line dependent on GM-CSF or IL-3 [16]. It was kindly provided by Dr. Kitamura (The Institute of Medical Science, The University of Tokyo, Tokyo, Japan) and maintained in RPMI 1640, 10% FCS, 10 ng/ml GM-CSF at 37°C in an atmosphere of humidified air containing 5% CO_2. Before use, TF-1 cells were washed three times in RPMI 1640 and 10% FCS without GM-CSF.

2.5. Cell growth

Cell growth was evaluated by the MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; Sigma, St. Louis, MO, USA) [17]. Briefly, 5 μg/ml of MTT was added to each well and incubated for 2 h. After formation of formazan crystal by viable cells, 100 μl of isopropanol/HCl was added to dissolve the crystals. The absorbance was measured at 550 nm using a microplate spectrophotometer. Monocytes (1×10^4/well) or TF-1 cells (2×10^4/well) were incubated for 3 days in a 96-well microplate (Falcon, Franklin Lakes, NJ, USA) with various concentrations of GM-CSF or IL-3 (0, 0.625, 1.25, 2.5, 5, and 10 ng/ml) and the BALF from a PAP patient or a control subject (0, 12.5, 25, 50 and 100 μg/ml). The cells were observed under a phase-contrast microscope (Diaphot 300; Nikon, Tokyo, Japan).

2.6. ^{125}I-GM-CSF receptor binding assays

The binding assay of GM-CSF to the receptor was performed as previously described [18]. Briefly, TF-1 cells (5×10^5) were suspended in 1 ml of RPMI 1640 medium with 150 pM of ^{125}I-GM-CSF and various concentration of the BALF (0, 15.6, 31.3, 62.5, 125 and 250 μg/ml) from an IPAP patient for 90 min at 15°C. As a background reaction, the same reaction mixture was incubated with a 1000-fold excess amount of cold GM-CSF. The cell suspensions were centrifuged at $250\times g$ for 5 min to remove unbound ^{125}I-GM-CSF from the cells, washed three times with cold PBS, and the radioactivity incorporated into the cells was measured by a γ counter Wizzard 1470 (Wallac, Turku, Finland). In some experiments, the receptor binding assays were performed after TF-1 cells (1×10^6/ml) were preincubated with 250 μg/ml of IPAP-BALF or PBS at 15°C for 90 min, washed with cold PBS three times.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Percent binding inhibition of monoclonal anti-GM-CSF antibody (23B6) to GM-CSF by the BALF was measured with the ELISA system. A micro-ELISA plate (Nunc, Roskilde, Denmark) was coated overnight at 4°C with 100 μl of 0.5 μg/ml anti-hGM-CSF monoclonal antibody. After washing five times with PBS, the plate was treated with a blocking reagent (Stabilicoat; BSI Corp., Eden Prairie, MN, USA) at room temperature for 1 h. After removal of the blocking reagent, 50 μl of 25 ng/ml GM-CSF and 50 μl of various protein concentrations of the BALF (0, 62.5, 125, 250, 375 and 500 μg/ml) from PAP patients or normal subjects were incubated in wells at room temperature for 2 h. After washing with PBS, a peroxidase labeled anti-hGM-CSF polyclonal antibody was added to each well and the mixture was incubated at 37°C for 1 h. Color development was performed using TMB solution (Color Reagents; R&D, Minneapolis, MN, USA). The absorbance was measured using a microplate spectrophotometer Model 3550 (Bio-Rad, Hercules, CA, USA). TNFα, IL-6 and MCP-1 were assayed with corresponding commercial assay kits (TNFα and MCP-1, R&D Systems, Minneapolis, MN, USA; IL-6, Biosource, Camarillo, CA, USA). The percent inhibition was calculated with the following equation:

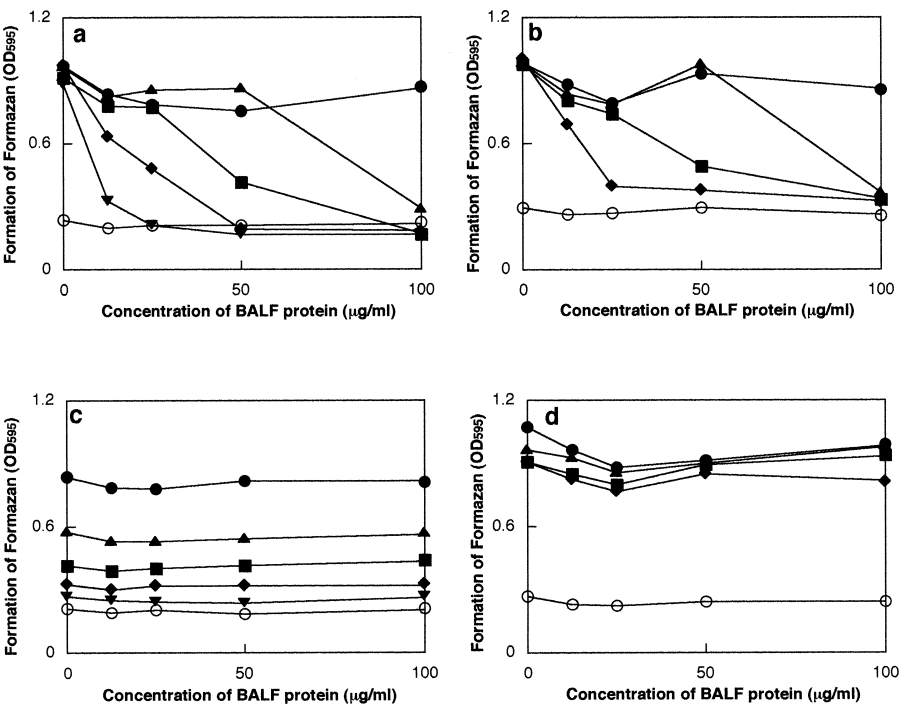

Fig. 1. The growth inhibition of TF-1 or monocytes by the BALF from a PAP patient. The horizontal axis is the concentration of BALF protein. (a: TF-1 incubated with GM-CSF; b: monocytes incubated with GM-CSF; c: TF-1 incubated with IL-3; d: monocytes incubated with IL-3). The vertical axis is the activity of viable cells examined by the MTT assay. Cells were incubated with various concentration of GM-CSF or IL-3 (10 ng/ml: ●; 5: ▲; 2.5: ■; 1.25: ◆; 0.625: ▼; 0: ○). In both TF-1 and monocytes, the MTT uptake was significantly decreased as the protein concentration of the BALF increased (a and b). In contrast, the BALF did not affect the bioactivity of IL-3 in both TF-1 and monocytes at all (c and d).

Percent inhibition (%) =

$$[1-(\text{detected GM}-\text{CSF (ng/ml)}/25\text{ (ng/ml)})]\times 100$$

2.8. Cross-linking studies

IPAP-BALF (500 μg/ml) or the BALF from a normal subject was incubated with ^{125}I-GM-CSF (6 nM) in the binding buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$) for 90 min at room temperature. As a background reaction, the same reaction mixture contained a 100-fold excess amount of cold GM-CSF together with IPAP-BALF. The cross-linking reaction was performed with 300 μM of disuccinimidyl suberate (DSS: Pierce, Rockford, IL, USA) at 4°C for 15 min. The reaction was quenched by adding 1 ml of quenching buffer (50 mM Tris-HCl pH 8.0, 15 mM NaCl, 2 mM EDTA). The mixtures were electrophoresed with SDS-polyacrylamide gel. The gel was dried and exposed to X-ray film.

3. Results

3.1. Inhibition of GM-CSF-dependent cell growth with IPAP-BALF

We found that IPAP-BALF inhibited the growth of monocytes and TF-1 cells when they were cultured with 1 ng/ml of GM-CSF (Fig. 1a,b). The inhibition was dependent on the concentration of the BALF added to the culture medium and was not observed when these cells were cultured with IL-3 instead of GM-CSF (Fig. 1c,d). BALF from a normal subject did not inhibit the growth of these cells cultured with 1n g/ml of GM-CSF. BALF from IPAP patients also reduced the viability of TF-1 cells as measured by suppression of MTT uptake. All of the 11 IPAP-BALF demonstrated this effect but the BALF from secondary PAP patients and normal subjects did not reduce the viability of TF-1 cells (Fig. 2).

TF-1 cells and blood monocytes were similar. As shown in Fig. 3, the viability of monocytes cultured with 1 ng/ml of GM-CSF and 100 μg/ml of IPAP-BALF for 72 h was less than 5%, whereas the viability was higher than 95% when monocytes were cultured with 1 ng/ml of GM-CSF and 100 μg/ml of the control BALF. These results indicate that the IPAP-BALF is not toxic to these cells but instead specifically inhibits the bioactivity of GM-CSF.

3.2. IPAP-BALF inhibits GM-CSF binding to TF-1 cells and to anti-GM-CSF antibody

We examined binding of ^{125}I-GM-CSF to TF-1 cells in the presence of IPAP-BALF to clarify whether loss of GM-CSF bioactivity is due to inhibition of cytokine/receptor interaction. As shown in Fig. 4a, GM-CSF binding to TF-1 cells was inhibited with the increased concentrations of BALF protein (Fig. 4a). The inhibition was not observed in BALF from a normal subject. This result indicates that the inhibition of bioactivity is due to blocking of cytokine/receptor interaction. Preincubation of TF-1 with IPAP-BALF did not influence binding of ^{125}I-GM-CSF to the cells; the binding profile of ^{125}I-GM-CSF to TF-1 cells preincubated with either the BALF or medium alone showed the same pattern (Fig. 4b). These results indicate that the IPAP-BALF does not interfere with the receptor directly.

We next examined binding of GM-CSF to a monoclonal antibody 23B6 specific for GM-CSF. We found that IPAP-BALF from all 11 patients interfered with the binding of GM-CSF to its antibody using a sandwich ELISA system. The percent inhibition was dose-dependent (Fig. 5). BALF from two secondary PAP patients or three controls showed no effect on binding of GM-CSF to antibody 23B6 even at a high protein concentration level up to 500 μg/ml. The IPAP-BALF did not affect binding of other cytokines including TNF, IL-6, and MCP-1 to corresponding antibodies (data not shown). Thus, these data suggest that the BALF specifically inhibits binding of GM-CSF to the monoclonal antibodies and receptors. This raises the possibility that the loss of GM-CSF bio-

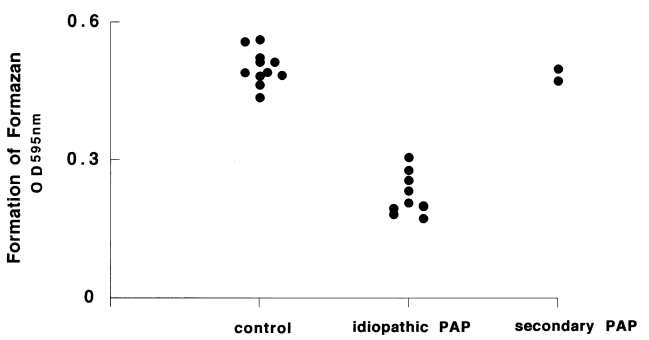

Fig. 2. Growth inhibition of TF-1 by the BALF from 10 controls, 11 IPAP patients, and two secondary PAP patients. The vertical axis is the optical density of formazan at 550 nm which corresponds to the activity of viable cells examined by the MTT assay. Cells were cultured with 1 ng/ml of GM-CSF and 100 μg/ml of the BALF in protein contents for 3 days.

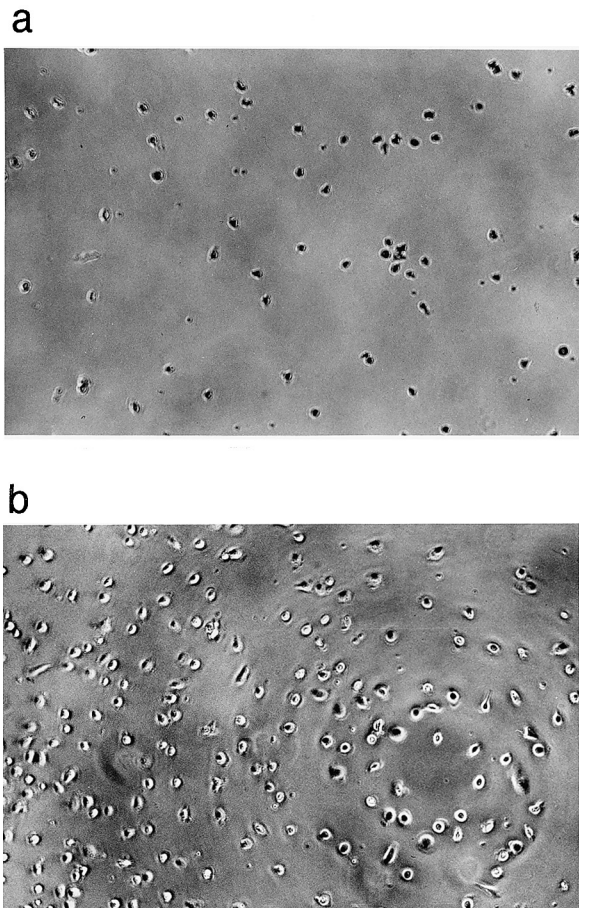

Fig. 3. Phase-contrast micrographs (low-power views, ×25) of monocytes incubated with 1 ng/ml of GM-CSF and 100 μg/nl of the BALF from an IPAP patient (a) or a control subject (b).

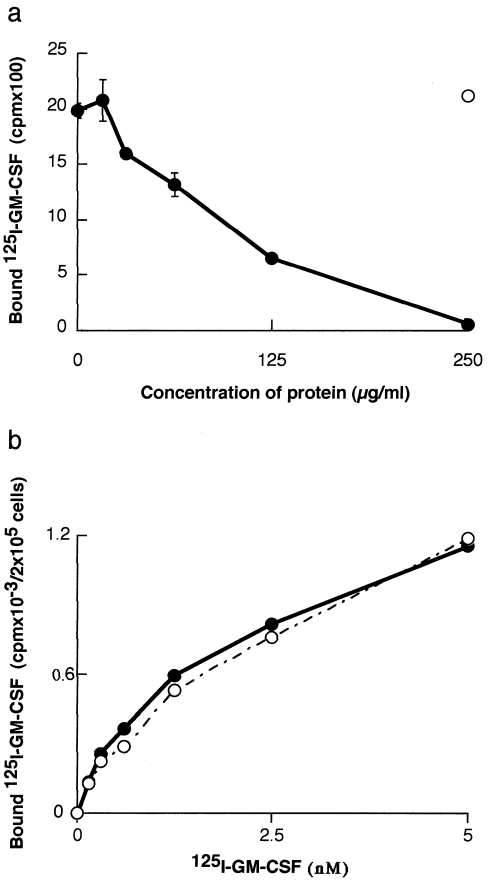

Fig. 4. a: Competitive inhibition of ^{125}I-GM-CSF binding to TF-1 cells by IPAP-BALF. TF-1 cells (5×10^5) were incubated with ^{125}I-GM-CSF (70 pM) and various concentration of IPAP-BALF (●) or 250 μg/ml of BALF from a control (○). ^{125}I-GM-CSF binding to TF-1 was inhibited with increased concentrations of the BALF protein. The data are shown as the mean ± S.D. b: The effect of preincubation of TF-1 cells with 100 μg/ml of IPAP-BALF on ^{125}I-GM-CSF binding to the cells (2×10^5). After TF-1 cells (1×10^6/ml) were preincubated with 250 μg/ml of IPAP-BALF (●) or PBS (○) at 15°C for 90 min, and washed with cold PBS three times, the cells were incubated with various concentrations of ^{125}I-GM-CSF (0–5 nM) at 15°C for 90 min.

activity is due to binding and neutralization of the cytokine itself.

3.3. Occurrence of ^{125}I-GM-CSF binding factor(s) in IPAP-BALF

We assayed for GM-CSF binding activity by cross-linking ^{125}I-GM-CSF to IPAP-BALF followed by SDS-PAGE size separation (Fig. 6). IPAP-BALF contained a ^{125}I-GM-CSF binding activity which produced two radioactive bands migrating at 39 and 41 kDa. Excess unlabeled GM-CSF significantly reduced the intensity of the bands and BALF from a control subject did not form these complexes. These data demonstrate that a factor in IPAP-BALF specifically binds to GM-CSF.

4. Discussion

In the present study, we have demonstrated the existence of a factor in the lung of IPAP patients that binds specifically to GM-CSF and neutralizes its bioactivity. IPAP-BALF inhibited the growth of blood monocytes and TF-1 cells, a GM-CSF/IL-3-dependent cell line. This inhibition was not due to non-specific toxicity of the IPAP-BALF since TF-1 cells supplemented with IL-3 continued to grow in the presence of IPAP-BALF. IPAP-BALF also prevented GM-CSF binding to TF-1 cells and to a monoclonal antibody specific for GM-CSF. Preincubation of TF-1 cells with IPAP-BALF followed by a medium change did not prevent GM-CSF binding suggesting that the factor expressed in IPAP did not bind the GM-CSF receptor. Cross-linking of radiolabeled GM-CSF to IPAP-BALF directly demonstrated that the factor expressed in IPAP bound GM-CSF. The inhibition of bioactivity or antibody binding was observed only in IPAP patients but not in secondary PAP patients or normal subjects, suggesting that this factor is involved in the pathogenesis of IPAP. Because BALF is a 100–500-fold diluted fluid of alveolar surfactant [19], GM-CSF binding activity in the lung of IPAP is estimated to be 100–500 times stronger than that observed in the BALF.

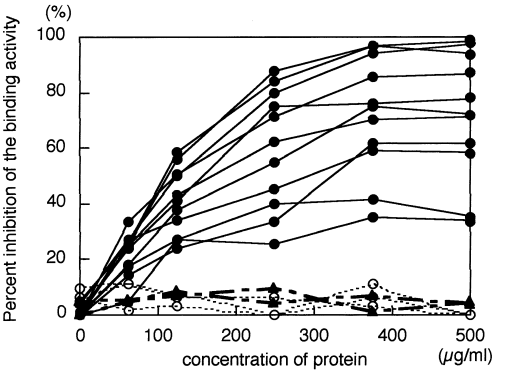

Fig. 5. Percent inhibition of the binding of monoclonal anti-GM-CSF antibody (23B6) to GM-CSF by the BALF from 11 idiopathic PAP patients (●), two secondary PAP (▲), and three control subjects (◦). The horizontal axis is the concentration of protein in BALF. The vertical axis is the percent inhibition of binding as described in Section 2.

Previously, soluble GM-CSF receptor α chain has been

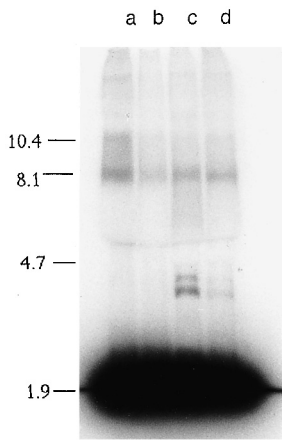

Fig. 6. Cross-linking of ^{125}I-GM-CSF to BALF followed by SDS-PAGE. Lane a: ^{125}I-GM-CSF without the BALF; lane b: ^{125}I-GM-CSF with the BALF from a normal subject; lane c: ^{125}I-GM-CSF with the BALF from an IPAP patient; lane d: ^{125}I-GM-CSF with the BALF from an IPAP patient with excess unlabeled GM-CSF. The bands at 39 and 41 kDa are due to a complex of ^{125}I-GM-CSF and a GM-CSF binding factor expressed in BALF from IPAP patients.

reported to be a specific ligand that bind to GM-CSF [20–22]. It is possible that soluble GM-CSF receptor may be overexpressed in the lungs of IPAP patients and inhibits the binding of GM-CSF to the receptor. However, our preliminary data showed that anti-GM-CSF receptor α chain neutralizing antibody failed to abrogate the GM-CSF binding activity (unpublished data). Thus, soluble GM-CSF receptor is unlikely to be a candidate for the GM-CSF binding factor in the lung of IPAP.

Mice deficient for GM-CSF or its receptor develop PAP [11,12]. Surfactant proteins were all significantly increased in BAL from both knockout mice compared with wild-type mice, and their lungs contained eosinophilic acellular material in alveolar spaces accompanied by increased tubular myelin. Correction of PAP in the receptor knockout mouse was accomplished by bone marrow transplantation from wild-type mice to βc-deficient mice demonstrating the central role of AM in this disorder [13]. Furthermore, Huffmann and colleagues recently reported that pulmonary epithelial expression of GM-CSF using an SP-C-GM-CSF construct in GM-CSF-deficient mice corrected PAP [23]. These model systems support a causal role for the GM-CSF neutralizing activity described in this investigation in the pathogenesis of human IPAP.

GM-CSF is a cytokine that increases complement- and antibody-mediated phagocytosis, enhances leukocyte chemotaxis, and augments antitumor immunity in macrophages. The lung produces GM-CSF constitutively and epithelial lining fluid in the alveolar spaces contains an approximately 100-fold higher concentration of GM-CSF than the plasma [24]. However, the true functions of GM-CSF in the lung are unknown. Previously, we demonstrated that GM-CSF in the lung promotes the proliferation of AM [25] but the number of AM is normal in the GM-CSF-deficient mouse. Thus, it is likely that GM-CSF does not contribute to the proliferation of AM in the steady state but instead modulates AM function. AM from GM-CSF knockout mice are filled with surfactant-like material. This phenotype improves after therapeutic lavage [26,27]. The data from the mouse model suggest that in the absence of GM-CSF, AM have a defect of surfactant catabolism. Therefore, the neutralization of GM-CSF bioactivity observed in this study is a plausible explanation of the pathogenesis of human primary PAP.

AM from GM-CSF knockout mice also have deficient chemotaxis. Because AM are important immune cells for first line defense in the lung, this may increase susceptibility to infections. In fact, IPAP is sometimes complicated by pulmonary opportunistic infections, particularly nocardiosis, *Pneumocystis carinii* pneumonia, and aspergillosis [28].

Wong et al. recently reported that mRNA of the GM-CSF gene in BAL cells from an IPAP patient is induced by lipopolysaccharide (LPS) stimulation in vitro, but that GM-CSF protein production was undetectable when the cells were incubated with or without LPS [29]. They also demonstrated that exaggerated release of IL-10 from PAP lung may suppress the production of GM-CSF. As IL-10 is known to suppress GM-CSF production at the transcriptional level by blocking NF-κB activation, this hypothesis does not clearly explain why mRNA of GM-CSF in the BAL cells is inducible but the production of protein is blocked. In contrast, as demonstrated in our study, the bioactivity of GM-CSF is blocked in the lungs of patients with IPAP by a factor that binds to GM-CSF. Further characterization of this factor will greatly contribute to the elucidation of not only the pathogenesis of IPAP but also the true function(s) of GM-CSF in the lung.

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