Isolation and Immunophenotyping of Mononuclear Cells from Human Lung Tissue

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Abstract

Objective To quantitatively isolate and immunologically phenotype mononuclear cells contained in human lung tissue.

Methods Normal appearing lung tissue as far distal to the resected lesion as possible was obtained from lung cancer patients. Lung tissue was thoroughly washed and cut into small pieces and digested with collagenase. Peripheral blood mononuclear cells (PBMNC) were prepared from controls using Ficoll gradient. Isolated cells and PBMNC were analyzed by flow cytometry. We immunohistochemically stained snap-frozen lung tissue with anti-CD3, CD4, CD8, CD20, and CD161 antibodies.

Participants Nineteen patients with lung cancer who underwent lobectomy were enrolled. Twelve healthy volunteers also participated as controls for flow cytometric analysis of PBMNC.

Results In forward scatter vs side scatter, 92.1±7.8% of isolated cells in the lymphoid population expressed leukocyte common antigen, CD45. The frequency of CD45-positive cells in the lymphoid population from lung tissue was as high as that from PBMNC (p=0.118). CD45-positive cells were successfully further extended by anti-CD3, CD4, CD8, CD19, and CD161 antibodies. Monocyte-macrophages bearing CD68 were also detected. CD68-positive alveolar macrophages dissapeared from alveolar spaces after thorough washing by immunohistochemical staining. Mononuclear cells in the interstitium were positively stained by anti-CD3, CD4, CD8, CD20, and CD161 monoclonal antibodies.

Conclusions We could isolate interstitial cells and analyze cell surface markers via flow cytometry from fresh lung specimens by collagenase digestion without further purification. Immunohistochemistry confirmed the presence of the cells detected by flow cytometry in the lung interstitium.

Key words: interstitial lung disease, lymphocytes, monocytes, collagenase, flow cytometry, immunohistochemistry

Introduction

Interstitial lung diseases (ILDs) represent a diverse group of pathological conditions involving the lung parenchyma. At present three methods [physiologic testing, high-resolution CT scanning, and bronchoalveolar lavage (BAL)] are useful for detection, staging, and following the course of ILD. BAL is a relatively noninvasive technique that may provide important information about the diagnosis and yield insights into immunologic, inflammatory, and infectious processes at the alveolar level (1). However, inflammatory-immune cells and their secretions out of the airway surface and the alveoli theoretically cannot be sampled by BAL. Immunohistochemistry of biopsy lung tissue helps researchers to phenotype lymphoid infiltrates, but it is difficult to characterize all of the cells contained in the affected lung tissue quantitatively.

Enzymatic digestion has been used to isolate interstitial lung cells. Holt et al reported a technique for the quantita-
Figure 1. Phenotypic characterization of mononuclear cells extracted from human lung tissue. A representative image of whole mononuclear cells sorted using the light scatters; forward-scatter and side-scatter, on a flow cytometry (A). Encircled regions R1 and R2 indicate the areas of lymphocytes and monocytes, respectively. An image of peripheral blood mononuclear cells (PBMNC) sorted by the same scatters is also shown as a control (B). After the first cell sorting using the light scatters, cells in the R1 were further sorted using fluorescence gating of anti-CD45 antibody (C). The frequency of CD45-positive cells in R1 was as high as that from the PBMNC sample (D). Gated cells were extended by three pairs of antibodies; anti-CD3 and CD19, anti-CD4 and CD8, and anti-161 and CD3, shown as representative images of flow cytometry, respectively (E, F, G). The numbers in the figure represent the percentages of fluorescence-positive cells in the corresponding areas. For detection of monocyte-macrophages, cells in R2 of the first sorting from human lung interstitium and PBMNC were analyzed by anti-CD68 antibody (H, I).

tive extraction of mononuclear cells from rat lung tissue using a mixture of collagenase and DNase followed by the simultaneous recovery and fractionation of cells on a six-step discontinuous percoll gradient (2). Salari et al successfully isolated human lung mast cells by digestion with a mixture of pronase, chymopapain, elastase, and collagenase followed by density gradient centrifugation (3). Watanabe et al successfully characterized mouse lung cells just by digestion with collagenase and a trypsin inhibitor and flow cytometry technique (4). To isolate and characterize interstitial cells contained in the human lung tissue quantitatively, we applied that method to surgically obtained human lung specimens. We also immunohistochemically confirmed the presence and localization of cells detected by flow cytometry.

Methods

Study subjects

Nineteen patients with non-small cell lung cancer were enrolled. Of the 19 patients, 5 were males and 14 were females with a mean age of 71 years ranging from 59 to 80. Heparinized peripheral blood was collected from 12 healthy volunteers as controls for flow cytometric analysis. All the
Figure 2. Comparison of CD68-positive alveolar macrophages in human lung specimens (A) before and (B) after washing with phosphate-buffered saline. Note that most of the alveolar macrophages (brown) disappeared from alveolar spaces after PBS washing. Original magnification was ×100.

Subjects gave their written informed consent to participate in this study. The research was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association, and the study was approved by the Committee of Ethics, Niigata University.

**Cell preparations**

Tissue was obtained from normal appearing lung as far distal to the resected lesion as possible in lobectomy specimens. Tissue cut into 3 parts was subjected to cell extraction, pathological examination, and immunohistochemistry. Interstitial cells were extracted from fresh human lung tissue following the previously described method with some modifications (4). One of the specimens was thoroughly washed with phosphate-buffered saline (PBS) to remove the remaining blood and cells including neutrophils, lymphocytes, and alveolar macrophages in the small airways and alveolar spaces. Washed lung tissue was scraped extensively with a pair of sterilized scissors into a homogenized suspension subjected to digestion with 0.5 mg/ml collagenase (WAKO, Osaka, Japan) and 0.1 mg/ml trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA) in minimum essential medium (Gibco BRL, Bethesda, MD, USA) in a 37°C shaking water bath for 30 minutes. The digested pieces of lung tissue and supernatant were passed through a 200-gauge stainless steel mesh to remove cell clumps and undissociated tissue. The filtered suspension was centrifuged and the pellet was resuspended in PBS at a concentration of approximately 2×10^6/ml. For preparation of peripheral blood mononuclear cells (PBMC), 10 ml of heparinized peripheral blood was mixed with 10 ml of PBS and layered onto the Ficoll gradients (Axis-Shield, Oslo, Norway) and centrifuged. About 1×10^7 mononuclear cells were collected and suspended in PBS at a concentration of 2×10^6/ml for flow cytometric analyses as a PBMC sample.

**Flow cytometry**

We performed flow cytometric analysis as previously described (5). Isolated cells from lung tissue and PBMC were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or pericin chloropyll protein (PerCP)- conjugated monoclonal anti-CD3, CD4, CD8, CD19, CD45, and CD161 antibodies (Becton-Dickinson, San Jose, CA, USA) in PBS for 20 minutes at 4°C. Stained cells were analyzed on flow cytometry (FACScan; Becton-Dickinson) using a Cell Quest program. Differentiation of lymphocytes was determined using flow cytometric analysis of light scatter chamber characteristics relating size and granulation, and fluorescence gating with anti-CD45 antibody. Three-color flow cytometry was then performed to calculate the percentages of CD3, CD4, CD8, CD19, and CD161 positive cells in a subset of CD45+ lymphocytes. Differentiation of monocyte-macrophages was also determined by light scatter chambers and staining by anti-CD68 antibody.

**Immunohistochemistry**

Immunohistochemistry was applied to fresh lung tissue as previously described (6). Five μm paraffin-embedded sections of lung tissue were first stained by incubation at 4°C overnight with the following monoclonal antibodies: anti-CD3, CD4, CD8, CD20 (Nichirei, Tokyo, Japan), CD68 (DAKO, Glostrup, Denmark), and CD161 antibody (Serotec, Oxford, UK) with the dilution rates recommended by the respective manufacturers. Sections washed with PBS were then incubated with rabbit anti-mouse IgG antibody for 30 min. After repeated washing with PBS, the product of the peroxidase reaction was revealed by 3-3' diaminobenzidine and hydrogen peroxide. The sections were lightly counterstained with hematoxylin and mounted on the slides.

**Statistical analysis**

The frequency of CD45-positive cells in the lymphoid
population in forward scatter vs side scatter between isolated mononuclear cells and PBMC was compared by Mann-Whitney U test. A P value less than 0.05 was considered significant.

### Results

**Isolation of mononuclear cells from human lung**

Interstitial cells extracted from lung tissue were sorted by flowcytometric analysis of light scatter chamber characteristics relating size and granulation (Fig. 1A). Although the flowcytometry image of isolated cells was different from that of PBMC (Fig. 1B), 92.1±7.8% of cells in the lymphoid population in forward scatter vs side scatter expressed...
Figure 4. Presence of natural killer cells and monocytes in the interstitium of human lung tissue. Specimens of normal lung parts were stained with anti-CD161 and CD68 monoclonal antibodies. Square outlined areas in panels A and C are shown as a higher magnification view in panels B and D, respectively. Positive cells are indicated by arrows. Original magnification, × 100 (A and C) and × 400 (B and D).

Table 1. Immunophenotypes of Mononuclear Cells Obtained from Human Lung Tissue

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Frequency (%)</th>
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<tbody>
<tr>
<td>Lymphocyte</td>
<td></td>
</tr>
<tr>
<td>CD3⁺</td>
<td>60.8±22.2</td>
</tr>
<tr>
<td>CD19⁺</td>
<td>1.3±1.1</td>
</tr>
<tr>
<td>CD4⁺</td>
<td>32.6±16.2</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>40.4±10.8</td>
</tr>
<tr>
<td>CD161⁺</td>
<td>41.0±11.8</td>
</tr>
<tr>
<td>CD3⁺CD161⁺</td>
<td>27.0±15.7</td>
</tr>
<tr>
<td>CD5⁺CD161⁺</td>
<td>14.0±6.5</td>
</tr>
<tr>
<td>Monocyte-macrophage</td>
<td></td>
</tr>
<tr>
<td>CD68⁺</td>
<td>46.1±12.6</td>
</tr>
</tbody>
</table>

Mean ± standard deviations are shown.

leukocyte common antigen (LCA, CD45, Fig. 1C). The frequency of CD45 positive cells on the lymphoid population from isolated mononuclear cells was not statistically different from that from PBMC (91.5±12.5%, p=0.118, Fig. 1D). These results indicate that we isolated lymphocytes from the lung interstitium by this technique as well as from peripheral blood.

**Phenotypic characterization of isolated mononuclear cells**

CD45⁺ cells were further sorted into pairs of the following monoclonal antibodies; anti-CD3, CD4, CD8, CD19, and CD161 antibody (Fig. 1E, F, G). CD45⁺ cells were clearly extended by all pairs of these antibodies. Mean and standard deviations of each phenotype of lymphocytes from lung tissue are summarized in Table 1. A quarter of the extracted lymphocytes did not express either CD4 or CD8 suggesting that these CD45⁺ positive cells were natural killer (NK) cells. Isolated cells in R2 were then analyzed by the expression of CD68. The frequency of CD68⁺ positive cells was higher than that of PBMC, but the signal of the molecule was less intensive (Fig. 1H, I).

**Immunohistochemical staining**

Specimens of paraffin-embedded lung tissue were immunohistochemically stained to locate lymphocytes and monocyte-macrophages expressing each CD antigen in the lung interstitium. At first, we examined the effect of PBS washing by immunostaining alveolar macrophages by anti-CD68 antibody (Fig. 2). Most of the CD68⁺ cells disappeared from the alveolar spaces of washed specimens.
(Fig. 2B). Then we located lymphocytes and monocyte-macrophages in the interstitium out of the alveolar areas. T lymphocytes were predominantly located in the perivascular and perilymphangial area with no difference in location between CD4+ and CD8+ cells (Fig. 3A-F). Most of the CD20+ cells were observed in the lymphocytic foci (Fig. 3G, H). CD161+ cells were located predominantly in the perivascular, perilymphangial, and pleural areas (Fig. 4A, B). CD68-positive cells were distributed both in alveolar spaces and in the interstitium corresponding to alveolar macrophages and tissue macrophages, respectively (Fig. 4C, D).

Discussion

A few techniques have been developed to isolate interstitial cells. Cells in the lung interstitium were mechanically and enzymatically dispersed and single cell populations of interest were then partially purified by gradient centrifugation for further experiments (2, 3). Flow cytometry is a technique that enables rapid and reliable quantification of various cellular characteristics including cell size and shape. It can be used for specific typing and quantification of lymphocyte subsets (7). For accurate qualitative and quantitative immunophenotyping via flow cytometry, cells must be isolated from fresh tissue. We extracted human lung mononuclear cells with collagenase and trypsin inhibitor for flow cytometric analysis. Holt et al reported that yields in the order of 10^8 viable mononuclear cells per gram of lung were routinely achieved with tissues from rat, mouse, and guinea-pig using collagenase digestion and gradient centrifugation (2). Although we did not calculate cell counts, we might expect similar yields from the human lung. Since we digested lung tissue by collagenase, the recovered cells should contain alveolar epithelial cells, peripheral airway epithelial cells, and endothelial cells in addition to mononuclear cells. The difference of cell components and contamination of cell debris and connective tissue in the sample may make the flowcytometry image of isolated cells look different than that of PBMC. Flow cytometry analysis revealed that mononuclear cells in the lymphocyte area were phenotyped CD45 positive, just like those from peripheral blood. The results suggest that it is possible to isolate lymphocytes with other lung cells from human lung tissue and immunologically phenotype them without purification by gradient centrifugation.

BAL has been an important technique in enhancing the understanding of mechanisms of lung injury, inflammation, repair, and fibrosis in many pulmonary diseases (1, 8). In ILDs, the right middle lobe or lingula is lavaged most commonly because the anatomy favors maximal recovery of fluid and cells from these sites when the patient is supine. Lavage in that site may provide a representative picture of the inflammatory and immune processes in the alveoli (9). The lung parenchyma damage in ILD involves predominantly or exclusively the lower and peripheral lung zones (10, 11). Thus, the information obtained from the right middle lobe or lingula by BAL might not accurately reflect the lung parenchyma damage in ILD. ILDs are generally characterized by a marked increase of inflammatory cells comprising macrophages, lymphocytes, neutrophils, and eosinophils in the alveolar walls and on the alveolar epithelial surface. However, the inflammation in ILDs often involves not only the alveolar walls and epithelium but also the capillary endothelium, the spaces between these structures, and the perivascular and lymphatic tissues. Cells located in the interstitium outside of the alveolar space or airway surface could not be sampled by the conventional BAL technique. Other methods for accurate evaluation of inflammatory cells present in this interstitial zone are needed. We found that most of the alveolar macrophages disappeared from the alveolar spaces after PBS washing. The result suggests that mononuclear cells seen in the immunohistochemistry may correspond to the cells extracted by this technique. We believe that the method described in this study may be a promising technique for accurate evaluation of lung interstitial cells.

Histological examination including immunohistochemical study of lung biopsy specimens has enabled identification of the in situ location of mononuclear cells in the lung interstitium and allowed simple phenotyping of lymphoid infiltrates. We immunohistochemically localized mononuclear cells with specific phenotypes detected by flow cytometry and confirmed that these cells were actually present in the lung interstitium out of the airway surface. By combining these two methods, histological study and phenotyping of isolated cells, we will be able to evaluate inflammatory cells of specific phenotypes in ILDs in a more quantitative manner. In addition to phenotypically characterizing mononuclear cells in the inflammatory lung, specific cellular components can be purified from isolated lung cells. Further in vitro study using purified cells may be promising to investigate the inflammatory mechanisms of ILD.

This study has some limitations. Lung tissue was collected from patients with lung cancer, which may influence the phenotypes of lymphocytes. We found about 30% of natural killer (NK) cells in lymphocytes from lung interstitium. This increase of NK cell frequency may well have been caused by the presence of lung cancer in the same lobe. PBMC or BAL lymphocytes from the same individual were not analyzed by flow cytometry. A comparative study of lymphocyte phenotypes between BAL and lung interstitium from the same patient with ILD is underway.

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References


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