Idiopathic pulmonary fibrosis (IPF) is a chronic inflammatory disorder restricted to the lungs. Leukocyte entry into the area of inflammation is regulated, at least partly, by endothelial expression of leukocyte-selective cell adhesion molecules (CAMs). To investigate the relevance of these CAMs to the accumulation of leukocytes in IPF, we examined the expression of E-selectin (endothelial leukocyte adhesion molecule-1; ELAM-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) by immunohistochemistry in lung tissue from nine patients with IPF and five nonsmoking normal subjects. The results demonstrated that in normal lungs, ICAM-1 was weakly expressed on endothelial cells, but neither E-selectin nor VCAM-1 was detected. In the lungs of patients with IPF, E-selectin expression on endothelial cells was restricted to honeycombing regions. Endothelial expression of ICAM-1 was increased throughout the tissue, but VCAM-1 was not detected in IPF. The distribution of leukocytes in lungs with IPF consisted of mostly lymphocyte accumulation in the interstitium and neutrophil accumulation within the airspaces of honeycombing regions. These results suggest that E-selectin may play a role in the recruitment of neutrophils in regions of honeycombing and that ICAM-1 may play a role in lymphocyte recruitment into the interstitium in addition to contributing to neutrophil recruitment in regions of honeycombing in patients with IPF.

Key words: E-selectin; idiopathic pulmonary fibrosis; intercellular adhesion molecule-1; vascular cell adhesion molecule-1

Idiopathic pulmonary fibrosis (IPF) is a chronic inflammatory disorder, limited to the lung, characterized by the presence of alveolitis and interstitial pneumonitis, damage to parenchymal epithelial cells, and abnormal accumulation of collagen. Although the etiology of IPF has not been clarified, there are reports that suggest that neutrophils play an important role in the pathogenesis. A significant increase of neutrophils in bronchoalveolar lavage (BAL) has been demonstrated in patients with IPF, and these neutrophils are known to be inflammatory effector cells that release mediators such as oxygen radicals, arachidonic acid metabolites, and proteolytic enzymes. Thus, these inflammatory mediators may cause parenchymal injury of the lung. However, in contrast to the findings in lavage fluid, pathologic studies have consistently failed to demonstrate significant numbers of neutrophils in patients with IPF. Neutrophils are rarely seen in the interstitium or airspaces, except in the lumen of distorted small airways. The exact source of the neutrophils in BAL remains unknown. Recent evidence indicates that leukocyte adhesion to endothelial cells is an indispensable step in the development of inflammatory disorders and that leukocyte entry into areas of inflammation is regulated, at least in part, by endothelial expression of leukocyte-selective cell adhesion molecules (CAMs). Each endothelial CAM preferentially (though not exclusively) recognizes a subset or subsets of leukocytes. E-selectin (endothelial leukocyte adhesion molecule-1; ELAM-1) belongs to the selectin family and mainly mediates the adherence of neutrophils. Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin gene superfamily and is expressed on a variety of cell types. It has been implicated in the adhe-
sion of all leukocytes, including lymphocytes and neutrophils. Vascular cell adhesion molecule-1 (VCAM-1) also belongs to the immunoglobulin gene superfamily, and mediates the adherence of lymphocytes, but not neutrophils.

In the present study, we examined the expression of E-selectin, ICAM-1, and VCAM-1 in the lungs of patients with IPF to investigate the relevance of these CAMs to the accumulation of leukocytes in IPF by immunohistochemical technique.

**METHODS**

**Patients**

Nine patients with IPF were studied. Four of the nine were also diagnosed as having lung cancer at the time of biopsy. All specimens were obtained by open lung biopsy. No patients had received any previous steroid therapy. None of the patients had a history of occupational exposure. The diagnosis of IPF was made by medical history, physical examination, laboratory test, chest radiograph, pulmonary function tests, and arterial blood gas analyses and was confirmed by histologic examination. Clinical data for each patient are shown in Table 1. Histologically normal lung tissue obtained from five nonsmoking patients who underwent operation for lung cancer was also investigated. All these specimens were obtained under informed consent.

**Immunohistochemistry**

Each lung specimen was immediately fixed in periodate-lysine 4% paraformaldehyde for 6 h, and washed in phosphate-buffered saline (PBS) solution containing increasing concentrations of sucrose. The fixed specimens were embedded (Tissue-Tec OCT Compound, Miles Pharmaceutical, Naperville, Ill.), frozen in dry ice ethanol, and serial sections of 6 μm were made by cryostat microtome. Monoclonal antibody (MoAb) BBIG-E6 for E-selectin, BBIG-I1 for ICAM-1, and BBIG-V1 for VCAM-1 were purchased from the manufacture (British Biotechnology Products Ltd, Cosmo Bio Co, Tokyo, Japan) and were used as first antibodies at the optimal concentration of 1.0 μg/mL, 0.2 μg/mL, and 1.0 μg/mL, respectively. Goat antimouse F(ab')2 fragments of IgG labeled with horseradish peroxidase (HRP) (Cosmo Bio Co, Tokyo, Japan) were used as the second antibody at a dilution of 1:100.

Cryostat sections to be observed by light microscopy were treated with 100% methanol containing 0.03% hydrogen peroxidase to inactivate endogenous peroxidase. Then, indirect HRP-labeled antibody method was applied for the immunologic reaction as previously described. Briefly, the procedure involves successive incubations with the first antibodies for 12 h at 4°C and the second antibody for 6 h at 4°C. They were then reacted with 0.25% diaminobenzidine (DAB) solution containing 0.01 M sodium azide and 0.01 M hydrogen peroxide, and counterstained with methyl green. For negative controls, we replaced the primary antibodies with PBS or another indifferent antibody of CD 56 (Immunotech S.A., anti-NCAM MoAb). No staining was observed in blood vessels, epithelial cells, or alveolar macrophages in these negative controls.

For electron microscopy, cryostat sections were treated similarly through the antibody incubation steps except that the endogenous peroxidase inactivation was omitted. The sections were fixed in 0.5% glutaraldehyde, washed, and then immersed sequentially in 0.25% DAB solution and in 0.25% DAB solution containing 0.01 M hydrogen peroxide. They were washed, and reacted with 2% osmium tetroxide in PBS, dehydrated in graded ethanol, and embedded in epoxy resin (Epon). Ultrathin sections, stained with lead citrate, were viewed with an electron microscope (Hitachi H-600).

**Evaluation**

At least three frozen blocks were cut and examined for each patient. We investigated the expression of these CAMs about vascular cells, including small vessels and capillaries, and nonvascular cells, including epithelial cells and alveolar macrophages. To confirm cell types present in each section, consecutive sections were stained with hematoxylin-eosin. The expression of each CAM was graded into the following four degrees by the intensity of staining: −, negative; ±, weakly stained; +, moderately stained; and ++, strongly stained. For each specimen examined, the percentage of cells stained with at least weak intensity was quantitated according to the following 0- to 4-point scale: 0 to 5% of cells were stained; 1, 6 to 25% of cells were stained; 2, 26 to 50% of cells were stained; 3, 51 to 75% of cells were stained; and 4, 76 to 100% of cells were stained. The evaluation of the biopsy specimens was done by two of us (A.N. and Y.T.) who were blinded as to the tissues examined.

**RESULTS**

**Histologic Findings**

All IPF tissues examined showed the common histologic features of usual interstitial pneumonia (UIP). The specimen demonstrated a complex mixture of normal lung parenchyma, regions of early fibrosis, and end-stage scarred lung, in a patchy and nonuniform distribution. We identified the expres-
sion of CAMs in three different regions for each specimen: (1) normal lung; (2) regions of interstitial pneumonia/interstitial pneumonitis characterized by mild to moderate thickening of alveolar septae with inflammatory cell infiltration of interstitium and proliferation of regenerating type 2 alveolar lining cells; and (3) honeycomb regions characterized by severe dense fibrotic change of lung parenchyma and markedly distorted or widened airspaces covered with metaplastic epithelium.

Leukocyte infiltration was observed in regions of both interstitial pneumonitis and honeycombing. The degree of leukocyte infiltration was moderate, consisting mostly of lymphocytes, with occasional monocytes and eosinophils. In scarred honeycomb regions, fewer lymphocytes were noted. However, significant numbers of neutrophils were seen in the airspaces.

**CAM Expression**

Figure 1 shows a normal lung tissue obtained from a nonsmoking normal subject that was immunohistochemically stained with MoAb for CAMs. In normal lungs, one half to three quarters of small vessels exhibited weak staining for ICAM-1, but alveolar capillaries were mostly negative for ICAM-1 (Fig 1, top). Alveolar epithelial cells expressed moderate ICAM-1 immunoreactivity on the membranes (Fig 1, top). Most alveolar macrophages were negative for ICAM-1. E-selectin and VCAM-1 were not detected on either endothelial cells or nonvascular cells in normal lung tissues (Fig 1, center and bottom). In normal lung parenchyma of patients with IPF, the staining pattern of these CAMs was similar to that of normal lungs. Thus, small vessel stained weakly positive and alveolar epithelial cells stained moderately positive for ICAM-1. E-selectin and VCAM-1 were not expressed at all (data not shown).

A representative staining pattern of a region of interstitial pneumonitis in patients with IPF is shown in Figure 2. In regions of pneumonitis, more than three quarters of the small vessels were moderately stained with ICAM-1 (Fig 2, top). Although alveolar capillaries were decreased in number, they were also reactive for ICAM-1 (Fig 2, top). The regenerating alveolar lining cells that were considered to be type 2 alveolar epithelial cells were strongly stained with ICAM-1 on the membranes (Fig 2, top). Alveolar macrophages forming clusters in alveolar spaces also stained for ICAM-1 (Fig 2, top). However, E-selectin was negative or only faintly present on vascular endothelial cells, and nonvascular cells did not stain for E-selectin (Fig 2, center). VCAM-1 was not expressed on either endothelial cells or nonvascular cells (Fig 2, bottom).

**Figure 1.** Normal lung tissues immunohistochemically stained with MoAb for (top, A) ICAM-1, (center, B) E-selectin, and (bottom, C) VCAM-1. Top, A, Small vessel endothelial cells (arrow) are weakly and alveolar epithelial cells (arrowheads) are moderately stained with ICAM-1. Center, B, and bottom, C, E-selectin and VCAM-1 are not stained (original magnification X345). Bar=50 μm.

A typical immunohistochemical staining pattern in honeycomb regions in patients with IPF is shown in Figure 3. In honeycomb regions, one half to three quarters of capillaries and more than three quarters of small vessels exhibited moderate staining for ICAM-1 (Fig 3, top). Metaplastic epithelial cells and alveolar macrophages were also moderately stained with ICAM-1. The most conspicuous feature of honeycomb regions is the expression of E-selectin on
endothelial cells with approximately one half to three quarters of small vessels staining moderately while most capillaries were negative (Fig 3, center). E-selectin was not detected on nonvascular cells. VCAM-1 was not expressed on either vascular or nonvascular cells in honeycomb regions (Fig 3, bottom). A case of lymphocytic interstitial pneumonia (LIP) with positive staining for VCAM-1 on follicular dendritic cells served as a positive control for our VCAM-1 stain (data not shown).

The expression of E-selectin on endothelial cells of honeycomb regions was further studied using immunoelectron microscopy that localized E-selectin ex-
Expression to the luminal plasma membrane of endothelial cells of small vessels (Fig 4).

Blood vessel expression of CAMs is summarized in Table 2. Expression on nonvascular cells is summarized in Table 3. There was no significant difference in the CAM expression in either the intensity of staining or the quantity of stained cells between smokers and nonsmokers in IPF lungs. All controls were nonsmokers, however. All nine cases showed a similar staining pattern for these CAMs regardless of the presence or absence of lung malignancy.

**Discussion**

In the present study, we have demonstrated that E-selectin is expressed on the pulmonary vascular endothelium in honeycomb regions of patients with IPF. E-selectin expression was not detected in normal lungs at all. Previous observations in animals revealed E-selectin expression on vascular endothelium in antigen-induced airway inflammation and in immune complex-induced lung inflammation. This indicates that E-selectin is not expressed in normal situation but is rapidly upregulated by stimulation. Further, MoAb to E-selectin blocked both the influx of neutrophils and the lung injury in the animal model of antigen- or immune complex-induced lung injury. These findings support the concept that E-selectin expression may be a crucial part of both neutrophil influx and the overall inflammatory process in lung. The limitation of E-selectin expression to the honeycomb regions may explain the limitation of neutrophil distribution in pathologic studies to these same regions. Hammar\(^7\) stated in his review that open lung biopsy specimens from patients with pulmonary fibrosis only rarely contain neutrophils in the interstitium or airspaces. When neutrophils were observed, they were usually seen in distorted small bronchi, probably secondary to fibrosis. In our study, neutrophil accumulation was limited to cystic airspaces within honeycomb regions in IPF lungs. These findings suggest that E-selectin-related neutrophil accumulation occurs only in honeycomb regions in IPF. Despite this limited distribution, we speculate that neutrophil accumulation may contribute to the formation of end-stage histologic changes rather than representing purely a secondary phenomenon. The most conspicuous histologic feature in IPF is the variability of findings in adjacent regions of lung with regions of normal lung, interstitial pneumonitis, and

**Table 3—Expression of Cell Adhesion Molecules on Epithelial Cells and Alveolar Macrophages in Lungs of Normal Subjects and Patients With Idiopathic Pulmonary Fibrosis**

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>E-Selectin</th>
<th>ICAM-1</th>
<th>VCAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal lung</td>
<td>-* (0)(^l)</td>
<td>- (0)</td>
<td>- (0)</td>
</tr>
<tr>
<td>Alveolar capillaries</td>
<td>- (0)</td>
<td>± (3)</td>
<td>- (0)</td>
</tr>
<tr>
<td>Small vessels</td>
<td>- (0)</td>
<td>± (3)</td>
<td>- (0)</td>
</tr>
<tr>
<td>IPF lung</td>
<td>- (0)</td>
<td>± (3)</td>
<td>- (0)</td>
</tr>
<tr>
<td>Normal lung parenchyma</td>
<td>- (0)</td>
<td>± (3)</td>
<td>- (0)</td>
</tr>
<tr>
<td>Alveolar capillaries</td>
<td>- (0)</td>
<td>± (3)</td>
<td>- (0)</td>
</tr>
<tr>
<td>Small vessels</td>
<td>- (0)</td>
<td>+ (4)</td>
<td>- (0)</td>
</tr>
<tr>
<td>Regions of interstitial pneumonitis</td>
<td>- (0)</td>
<td>+ (4)</td>
<td>- (0)</td>
</tr>
<tr>
<td>Alveolar capillaries</td>
<td>- (0)</td>
<td>+ (4)</td>
<td>- (0)</td>
</tr>
<tr>
<td>Small vessels</td>
<td>- (0)</td>
<td>+ (4)</td>
<td>- (0)</td>
</tr>
<tr>
<td>Regions of honeycombing</td>
<td>- (0)</td>
<td>+ (3)</td>
<td>- (0)</td>
</tr>
<tr>
<td>Capillaries</td>
<td>+ (3)</td>
<td>+ (3)</td>
<td>- (0)</td>
</tr>
<tr>
<td>Small vessels</td>
<td>+ (3)</td>
<td>+ (4)</td>
<td>- (0)</td>
</tr>
</tbody>
</table>

*Intensity of staining: -, negative; ±, weakly stained; +, moderately stained.
\(^l\)Quantity of stained cells: 0, 0 to 5% of cells stained; 1, 6 to 25% of cells stained; 2, 25 to 50% of cells stained; 3, 51 to 75% of cells stained; 4, 76 to 100% of cells stained.

\(\dagger\)Intensity of staining: -, negative; +, moderately stained; ++, strongly stained.
\(^1\)Quantity of stained cells: 0, 0 to 5% of cells stained; 1, 6 to 25% of cells stained; 2, 25 to 50% of cells stained; 3, 51 to 75% of cells stained; 4, 76 to 100% of cells stained.
honeycombing occurring in a haphazard pattern.\textsuperscript{5,7} Consequently, an increase in lavage neutrophils in patients with IPF may reflect E-selectin expression in scattered regions of microscopic honeycombing.

VCAM-1 is not expressed in the absence of inflammatory stimulation, but is induced strongly on endothelial cells by inflammatory mediators such as tumor necrosis factor-\(\alpha\), interleukin 1, or lipopolysaccharide.\textsuperscript{18} VCAM-1 binds lymphocytic and monocytic cell lines via the \(\beta 1\) integrin VLA-4,\textsuperscript{10} which is present on most leukocytes with the exception of neutrophils.\textsuperscript{18} The present study failed to reveal VCAM-1 expression in normal lungs or in the lungs of patients with IPF. This suggests that VCAM-1 might not contribute to leukocyte influx in the lungs of patients with IPF.

The expression of ICAM-1 on vascular endothelial cells in IPF was increased compared with normal subjects. ICAM-1 has been characterized as a cell surface ligand for the lymphocyte function-associated antigen adhesion receptor, and it contributes to lymphocyte adhesion.\textsuperscript{16} In contrast to E-selectin expression, increased expression of ICAM-1 on endothelial cells was widely recognized in lung tissues from patients with IPF, including both regions of pneumonitis and honeycombing. Leukocyte infiltration in the interstitium consisted mainly of lymphocyte accumulation. Therefore, our results indicate that ICAM-1, but not VCAM-1, appears to play a key role in the recruitment of lymphocytes in IPF.

ICAM-1 has also been shown to mediate the adherence of neutrophils to endothelial cells.\textsuperscript{17} Anti-ICAM-1 MoAb reportedly inhibited migration of neutrophils to the phorbol ester-induced inflammatory lung.\textsuperscript{26} Recently, Butcher\textsuperscript{27} has suggested a general model of leukocyte-endothelial cell interaction consisting of at least three sequential events. In this model, E-selectin or other selectins first mediate the primary adhesion, and then ICAM-1 mediates the sustained adhesion of neutrophils to endothelial cells. The staining for serial sections of the lungs in our study revealed concurrent expression of E-selectin and ICAM-1 only in honeycomb regions but not in regions of pneumonitis/alveolitis (Figs 2 and 3). Taking previous observations and the present results into consideration, we speculated that neutrophil accumulation in the lungs of the patients with IPF may occur as a result of simultaneous expression of E-selectin and ICAM-1.

In our study, there was no significant difference in the CAM expression between nonsmokers and smokers in patients with IPF. Schwartz et al\textsuperscript{28} reported that cigarette smoking strongly influences BAL cellularity in patients with IPF. Since our study is restricted to endothelial CAMs, it may be possible that mechanisms other than these CAMs are involved in leukocyte recruitment in IPF lungs.

We observed strong expression of ICAM-1 on regenerating type 2 alveolar epithelial cells in IPF that was consistent with a recent report.\textsuperscript{29} Positive staining for ICAM-1 was also present on alveolar macrophages in patients with IPF. Previous observations demonstrated that ICAM-1 plays an important role in antigen presentation,\textsuperscript{30} and provides a costimulatory signal that is needed for T-cell activation.\textsuperscript{31} We reported previously the strong expression of major histocompatibility complex (MHC) class 2 antigen on regenerating type 2 alveolar epithelial cells and alveolar macrophages in patients with IPF, suggesting that regenerating type 2 alveolar epithelial cells and alveolar macrophages in patients with IPF may play a role as antigen-presenting cells.\textsuperscript{32} In this study, we showed that the expression of ICAM-1 was distributed similarly to MHC class 2 antigen in IPF. These results also support the role of regenerating type 2 alveolar epithelial cells and alveolar macrophages as antigen-presenting cells in patients with IPF.

ACKNOWLEDGMENTS: The authors thank Professor Hidehiko Saito, First Department of Internal Medicine, Nagoya University School of Medicine, for his valuable suggestions and comments during this work, and Dr. Tomohisa Shibaokami for preparing the tissue specimens. We also thank Dr. Takeshi Komatsu and Dr. Masashi Yamamoto for their helpful suggestions and support.

REFERENCES


9 Pober JS, Cotran RS. The role of endothelial cells in inflammation. Transplantation 1990; 50:537-44


11 Osborn L. Leukocyte adhesion to endothelium in inflammation. Cell 1990; 62:3-6

Ruco Davies


27 Butcher EC. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 1991; 67:1033-36


