The Role of Anti–Endothelial Cell Antibody–Mediated Microvascular Injury in the Evolution of Pulmonary Fibrosis in the Setting of Collagen Vascular Disease

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Abstract

We encountered 16 patients with connective tissue disease in whom pulmonary fibrosis developed. Routine light microscopic, ultrastructural, and direct immunofluorescent analyses were conducted, and circulating antibodies, including those of endothelial cell derivation, were assessed using indirect immunofluorescence and Western blot assays. Underlying diseases were dermatomyositis, scleroderma, mixed connective tissue disease, scleroderma in the presence of a Sjögren syndrome, rheumatoid arthritis, and anti-Ro–associated systemic lupus erythematosus. Antibodies to one or more Ro, RNP, Jo 1, OJ, and/or nucleolar antigens were seen in all cases and antiphospholipid antibodies in half. All biopsies revealed microvascular injury in concert with intraparenchymal fibrosis; in some cases, there were corroborative ultrastructural findings of microvascular injury. Patterns of fibroplasia represented nonspecific interstitial pneumonitis and usual interstitial pneumonitis. We noted IgG, IgA, and/or complement in the septal microvasculature. In 6 cases with available serum samples, indirect immunofluorescent endothelial cell antibody studies were positive and Western blot studies showed reactivity of serum samples to numerous endothelial cell lysate–derived proteins.

Pulmonary fibrosis, a recognized complication of systemic connective tissue disease, develops in connective tissue disease syndromes with pathogenetically established immune-based microvascular injury at other sites. A similar mechanism of antibody-mediated endothelial cell injury may be the basis of the tissue injury and fibrosing reparative response.

Connective tissue diseases (CTDs) represent a heterogeneous group of disorders of unknown cause that are accompanied by circulating autoantibodies that may evoke tissue injury. The pathogenetic dilemma associated with CTDs in general includes the following issues: (1) The mechanisms resulting in autoantibody formation are not clear. (2) There is no clear explanation for the specificity of the organ-specific cellular targets of the antibodies. (3) The pathogenetic consequence of these antibodies are unknown.

Pulmonary fibrosis (PF) is among the most serious sequelae associated with collagen vascular disease, defining an important cause of morbidity and mortality.1 There are select CTD syndromes in which this complication is more likely to develop, namely those conditions associated with multiorgan immune-based microvascular injury.2,3 These entities encompass scleroderma, dermatomyositis, mixed connective tissue disease (MCTD), scleroderma in the presence of a Sjögren syndrome, rheumatoid arthritis, and anti-Ro–associated systemic lupus erythematosus (anti-Ro SLE), and rheumatoid arthritis (RA).1,4-6 In fact, the incidence of PF is particularly high in the setting of scleroderma; up to 70% of patients with scleroderma manifest some degree of parenchymal fibrosis.7 As expected, one of the main causes of death in patients with dermatomyositis-polymyositis, MCTD, and scleroderma is respiratory dysfunction, reflecting relentless, progressive intrapulmonary fibroplasia.6,8

Although there is substantial literature linking PF to underlying collagen vascular disease, the basis of the fibrosis is undetermined. In contrast, the mechanism of extrapulmonary tissue injury in these conditions is better elucidated. Given the ubiquitous nature of the autoimmune-based microvascular injury operative in other organ sites such as the skin and muscle and the presence of circulating anti–endothelial cell
antibodies in these various syndromes, we hypothesized that these antibodies are an inciting trigger to PF.

Studies support the hypothesis that immune-based microvascular injury may be important in the propagation of PF. In particular, endothelial cell injury has been linked to idiopathic PF (IPF) and fibrosis developing in the setting of scleroderma. We found that patients with IPF and scleroderma who developed PF manifested deposition of complement and immunoglobulin within the septal microvasculature; there was also serologic evidence of circulating anti–endothelial cell antibodies. In regards to the latter point, Western blot studies showed reactivity to endothelial cell lysates of serum from patients with IPF and scleroderma. In support of this finding, Fujita and coworkers demonstrated reactivity of serum from patients with IPF and collagen vascular disease with cytokeratin 19, an important and unique constituent of pulmonary microvascular endothelial cells. Moreover, Ihn and coworkers showed a direct association between anti–endothelial cell antibodies and the development and severity of PF.

This article describes the clinical and pathologic findings in 16 patients with connective tissue disease syndromes in whom lung biopsy showed PF. By using direct (DIF) and indirect (IIF) immunofluorescence, electron microscopy, Western blot analysis, and serologic studies to assess for vascular cofactors, we explored a role for autoimmune-based microvascular injury.

Materials and Methods

Patient Population

Sixteen patients with an established history of underlying collagen vascular disease and clinical and radiographic features of PF underwent open lung or transbronchial biopsy during a 46-month period. In cases in which an open lung biopsy was performed, the area of suspected involvement was determined preoperatively by computed topographic studies. Specimens were selected from areas containing grossly altered parenchyma and more normal lung tissue when possible.

Routine Light Microscopy

The lung tissue specimens examined pathologically included paraffin-embedded, formalin-fixed tissue specimens stained with H&E, periodic acid–Schiff, and Masson trichrome preparations.

DIF Studies

All transbronchial and open lung biopsy specimens were received fresh. One portion was procured for routine light microscopic assessment, and another piece was snap frozen for purposes of DIF testing. The tissue sections were then incubated with commercially prepared fluoresceinated antisera specific for human IgG, IgM, IgA, C3, C1, C3d, C4d, and C5b-9. The samples were assessed under oil for optimal interpretation because the deposition pattern is typically very fine and may not be detected under x400 magnification.

C4d Immunofluorescent Assay

The tissue sample was cut at 2 to 4 µm thickness and placed on Fisher/Superfrost Plus slides; slides were allowed to air dry for 30 minutes followed by a rinse in phosphate-buffered saline (PBS). As a preblock, 150 µL of Avidin D (100 g/mL in PBS/1% bovine serum albumin) was applied for 20 minutes followed by a PBS rinse. Subsequently, 150 µL (10 g/mL in PBS) of d-biotin preblock was applied to the slide for 20 minutes followed again by a PBS rinse. The monoclonal antibody to C4d (clone 10-11) at a dilution of 1:100 (10 g/mL) was applied for 30 minutes followed by a PBS wash for 2 to 3 minutes. An antimouse IgG (H & L) diluted to a concentration of 1:100 was applied for 30 minutes, followed again by a PBS wash. Fluorescein isothiocyanate–streptavidin, 1:50 dilution, for 30 minutes was followed by a 2- to 3-minute PBS wash.

C5b-9 Assay

Mouse anti–C5b-9 (M777; DAKO) diluted at 1:25 in PBS was applied to cryostat sections for 45 minutes and then washed twice in PBS for 5 minutes, following which a fluorescein-conjugated antimouse immunoglobulin (F232; DAKO) diluted in PBS at 1:25 (DAKO) was applied and incubated for 30 minutes. Two additional 5-minute PBS washes were performed.

IIF Testing to Assess for Anti–Endothelial Cell Antibodies

In addition to DIF, we also applied an indirect method for diagnostic assessment. There were 2 principal methods of IIF testing. The first was the neonatal rodent lung assay, and the second used pulmonary endothelial cell and human umbilical vein endothelial cell (HUVEC) cytocentrifuged preparations. The IIF assay is most useful in certain select case scenarios, ie, IPF, collagen vascular disease, the setting of transplantation, and humoral allograft rejection.

Endothelial Cell Cytocentrifuge Assay

Serum samples were diluted to a dilution factor of 1:100 and incubated with acetone-fixed cytocentrifuged preparations of lung microvascular endothelial cells (LMVEC), HUVEC, and commercially prepared slides of human epithelial cells (HEp-2; INOVA Diagnostics, San Diego, CA). Antibody binding was detected with fluoresceinated goat antihuman IgG. Because of the role of anti–endothelial cell antibodies of the IgA isotype in RA, we also conducted the assay using goat antihuman IgA (dilution 1:100 in PBS; Caltag, Burlingame, CA).
Neonatal Rodent Lung Assay

The neonatal rodent lung assay was performed using a 3-day-old newborn rat. After the rat was killed, its lungs were removed. A whole mount of the lung was placed on TissueTek OCT medium (Sakura Finetek, Torrance, CA) and frozen. The block was then cut at 6 µm and placed on poly-L-lysine-coated slides. The slides were fixed in acetone and stored at –60°C. The lung tissue slides were placed in PBS for 5 minutes. A sample of the patient’s serum was applied to the neonatal lung tissue slide and incubated for 45 minutes. The slide was then washed in PBS twice, for 5 minutes each time. A fluorescein-conjugated goat anti-human IgG diluted at 1:20 in PBS was then applied to the slide. The slide was then incubated for 45 minutes and washed in PBS twice for 5 minutes.

Western Blot Studies

The method for the Western blot studies was described in an earlier report. Monolayers of LMYVEC and HUVENC and human embryonic lung fibroblasts (MRC5, American Type Culture Collection, Manassas, VA) were lysed with a sodium fluoride-containing buffer (Sigma Chemical, St Louis, MO) for 30 minutes at 4°C. An equal volume of loading buffer was added to each lysate before boiling. Electrophoresis through 10% sodium dodecyl sulfate–polyacrylamide gels was used to isolate the protein extracts. Following transfer to a nitrocellulose membrane (Osmonics, Westborough, MA), the membranes were incubated overnight at 4°C with a blocking solution. Patient serum was added to the blocking solution at a 1% concentration and again incubated overnight at 4°C on an orbital shaker. Blots were repeatedly rinsed in tris(hydroxymethyl)aminomethane-buffered saline before a 30-minute incubation with a horseradish peroxidase–conjugated goat anti-human IgG/IgM and/or IgA (Jackson ImmunoResearch Laboratories, West Grove, PA). Antibody complexes were detected using the Supersignal West Femto detection of IgG, IgM, and IgA. Serum samples, collected at room temperature, were used. The QUANTA Lite ACA (INOVA Diagnostics) enzyme-linked immunosorbent assay (ELISA) was used for semiquantitative detection of IgG, IgM, and IgA cardioliopin antibodies. An ELISA performed by Cambridge Biomedical (Brighton, MA) was used to detect antiphosphatidylserine antibodies and antiphosphatidylcholine of IgG, IgM, and IgA isotypes.

Electron Microscopy

After the initial fixation in 3% buffered glutaraldehyde, tissue fragments were washed twice with sodium cacodylate buffer and then postfixed in 1% osmium tetroxide in sym-colloidine buffer (pH 7.6) for 1 hour at room temperature. Following 2 washes with sym-colloidine buffer (10 minutes each) the tissue was en-bloc stained with a saturated aqueous uranyl acetate solution (pH 3.3) for 1 hour. Tissue fragments were dehydrated in a graded ethanol series up to absolute (10 minutes each). Acetone was used as the transitional solvent, with 2 changes for 10 minutes each. The tissue fragments were infiltrated overnight with a 1:1 mixture of acetone and Spurr epoxy resin (Electron Microscopy Sciences, Fort Washington, PA). Finally, the tissue pieces were placed into BEEM embedding capsules (BEEM, West Chester, PA) containing 100% Spurr resin. Polymerization of epoxy blocks was carried out at 70°C overnight.

Polymerized blocks were sectioned with an LKB Nova ultramicrotome (LKB, Bromma, Sweden). Methylene blue–basic fuchsin–stained semithin (750-nm) sections were evaluated. Ultrathin (80-nm) sections were collected on 200 mesh copper grids (Electron Microscopy Sciences) and poststained with uranyl acetate (15 minutes) and lead citrate (3 minutes). Electron micrographs were generated with a Zeiss EM 900 transmission electron microscope (Carl Zeiss SMT, Thornwood, NY) equipped with a MegaView III digital camera (Soft Imaging System, Münster, Germany).

Serologic Examination

In addition to the aforesaid Western blot and fluorescent methods to establish the presence or absence of circulating anti–endothelial cell antibodies, antibodies known to be associated with endothelial cell injury were evaluated and included anti-Ro/SSA, anti-ribonucleoprotein (anti-RNP) and certain of the myositis-associated antibodies such as anti–Jo 1, anti–PL7, anti–PL12, anti–EJ, anti–OJ, anti–Mi-2, anti–schistosomula-released products, anti–polymyositis-scleroderma, anti–Ku, anti–U1RNP, anti–U2RNP, and anti–Ro. In addition, a full antiphospholipid antibody (APAB) evaluation was conducted in light of the proffered role of these antibodies in the induction of endothelial cell injury. The antiphospholipid screen comprised an assessment of antibodies to β₂-glycoprotein, phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and anticardiolipin of all isotypes (IgG, IgM, and IgA).

DIF studies were conducted on samples from patients with other forms of pulmonary disease unrelated to collagen vascular disease. The neonatal lung assay was also conducted on samples from patients with CTD without lung disease, patients with other forms of lung disease, and patients with other systemic diseases in whom there was no pulmonary involvement. The IIF endothelial cell assay was performed on samples from patients with CTD without lung disease, and patients with other forms of pulmonary disease. Western blot studies were performed on samples from 5 healthy subjects and 4 patients with other forms of pulmonary disease. Western blot studies were performed on samples from 5 healthy subjects and 9 patients with other forms of lung disease.
Results

Clinical Results

The patient cohort comprised 16 patients (14 women and 2 men) with an average age of 47.7 years at the time of biopsy in whom there was an established history of collagen vascular disease Table 1. In all cases, a relatively recent onset of pulmonary symptoms and/or worsening symptoms led to a transbronchial biopsy in 6 patients or an open lung biopsy in the remainder. The primary symptoms were shortness of breath and cough. The duration of symptoms ranged from a few months to 1 year before biopsy.

The results of radiographic and clinical assessment were compatible with PF in all 16 cases. Among the radiographic findings were peripheral reticular densities and ground-glass opacities with bilateral posterior basilar accentuation of disease Image 1 and Image 2. In 2 patients with underlying dermatomyositis, there was concomitant upper lobe involvement. In 9 cases, the radiographic interpretation was one of usual interstitial pneumonitis (UIP) based on the degree of oblitative honeycomb fibrosis.

Pulmonary function studies revealed restrictive changes and a reduction in diffusion capacities. The most striking abnormalities were in patients with dermatomyositis and MCTD. There was immunosuppressive intervention in most patients, primarily with cyclophosphamide, mycophenolate, and prednisone. The patients with dermatomyositis-associated PF showed only marginal improvement. One patient died following the open lung biopsy; the remainder were alive at last follow-up (range, 6 months to 3 years from biopsy), but with continued respiratory compromise.

Among the underlying CTD syndromes were dermatomyositis in 7 patients, MCTD in 2, scleroderma in 2, sclerodermatomyositis in 1, anti-Ro-associated SLE in 1, RA in 2, and Sjögren syndrome (SS) in 1. The relevant serologic findings included anti-OJ and anti–Jo 1 antibodies in 2 patients with dermatomyositis; antibodies to RNP in both patients with MCTD; antibodies to Ro in 1 patient each with scleroderma, SS, and SLE; and antinucleolar antibodies in 1 patient each with sclerodermatomyositis, SS, and scleroderma. Three patients had positive rheumatoid factors—one patient with scleroderma and both patients with RA. Six patients had APABs, including antiphosphatidylcholine in 2, antiphosphatidylserine in 2, antiphosphatidylethanolamine in 5, and anticardiolipin in 1; there was a positive lupus anticoagulant in 1 and anticardiolipin antibodies in 1. One patient with APAB had SS with a history of deep venous thrombosis, with the first episode in 1976 and another in 1993.

The pathology results are given in Table 2.

Light Microscopic Findings

Transbronchial biopsy material was available in 6 cases; in the remainder of the cases open lung biopsy material was available for review. In 11 cases, there was homogeneous septal expansion accompanied by variable inflammation compatible with nonspecific interstitial pneumonitis (Image 2). This

<table>
<thead>
<tr>
<th>Case No.</th>
<th>FEV₁ (% Predicted)</th>
<th>FVC (% Predicted)</th>
<th>DLCO (% Predicted)</th>
<th>Serologic</th>
<th>Radiologic</th>
<th>Pathologic</th>
<th>Treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45.00</td>
<td>84.00</td>
<td>2700</td>
<td>RF 60</td>
<td>UIP</td>
<td>NSIP</td>
<td>Cyclophosphamide; prednisone</td>
<td>Marginal improvement; alive</td>
</tr>
<tr>
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<td>91.00</td>
<td>48.00</td>
<td>23.00</td>
<td>APAB</td>
<td>UIP</td>
<td>NSIP</td>
<td>Cyclophosphamide; prednisone</td>
<td>Marginal improvement; alive</td>
</tr>
<tr>
<td>3</td>
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<td>67.00</td>
<td>28.00</td>
<td>Anti-U1RNP and APAB</td>
<td>UIP</td>
<td>NSIP</td>
<td>Cyclophosphamide; prednisone</td>
<td>Significant improvement</td>
</tr>
<tr>
<td>4</td>
<td>49.00</td>
<td>53.00</td>
<td>NA</td>
<td>Antinucleolar; RF 339</td>
<td>UIP</td>
<td>UIP</td>
<td>Cyclophosphamide; prednisone</td>
<td>Significant improvement</td>
</tr>
<tr>
<td>5</td>
<td>41.00</td>
<td>38.00</td>
<td>NA</td>
<td>Anti–Jo 1 APAB</td>
<td>NSIP</td>
<td>UIP</td>
<td>Prednisone; cyclophosphamide</td>
<td>Stable</td>
</tr>
<tr>
<td>6</td>
<td>32.00</td>
<td>64.00</td>
<td>NA</td>
<td>RF</td>
<td>UIP</td>
<td>NSIP</td>
<td>Prednisone; cyclophosphamide</td>
<td>Worsening</td>
</tr>
<tr>
<td>7</td>
<td>92.00</td>
<td>97.00</td>
<td>80.00</td>
<td>Anti–Jo 1; anti-Ro APAB</td>
<td>NSIP</td>
<td>NSIP</td>
<td>Prednisone</td>
<td>Died after open lung biopsy</td>
</tr>
<tr>
<td>8</td>
<td>83.00</td>
<td>45.00</td>
<td>20.00</td>
<td>20.00 RF; anti-RNP APAB</td>
<td>NSIP</td>
<td>UIP</td>
<td>Prednisone</td>
<td>Died after open lung biopsy</td>
</tr>
<tr>
<td>9</td>
<td>95.00</td>
<td>95.00</td>
<td>56.00</td>
<td>Anti-Ro APAB</td>
<td>NSIP</td>
<td>UIP</td>
<td>Prednisone; cyclophosphamide</td>
<td>Worsening</td>
</tr>
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<td>10</td>
<td>56.00</td>
<td>52.00</td>
<td>44.00</td>
<td>Anti-Ro APAB</td>
<td>NSIP</td>
<td>UIP</td>
<td>NSIP</td>
<td>Interferon; prednisone</td>
</tr>
<tr>
<td>11</td>
<td>83.00</td>
<td>83.00</td>
<td>39.00</td>
<td>Antinucleolar; RF 13</td>
<td>UIP</td>
<td>UIP</td>
<td>Prednisone; cyclophosphamide</td>
<td>Stable</td>
</tr>
<tr>
<td>12</td>
<td>80.00</td>
<td>89.00</td>
<td>83.00</td>
<td>RF 36</td>
<td>NSIP</td>
<td>NSIP</td>
<td>Prednisone</td>
<td>Stable</td>
</tr>
<tr>
<td>13</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Anti–Ro APAB</td>
<td>NSIP</td>
<td>NSIP</td>
<td>Prednisone</td>
<td>Stable</td>
</tr>
<tr>
<td>14</td>
<td>60.00</td>
<td>66.00</td>
<td>75.00</td>
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<td>NSIP</td>
<td>NSIP</td>
<td>Prednisone</td>
<td>Worsening</td>
</tr>
<tr>
<td>15</td>
<td>58.00</td>
<td>64.00</td>
<td>48.00</td>
<td>Positive ANA</td>
<td>NSIP</td>
<td>UIP</td>
<td>Prednisone</td>
<td>Worsening</td>
</tr>
<tr>
<td>16</td>
<td>25.00</td>
<td>28.00</td>
<td>36.00</td>
<td>Positive ANA</td>
<td>NSIP</td>
<td>UIP</td>
<td>Prednisone</td>
<td>Worsening</td>
</tr>
</tbody>
</table>

ANA, antinuclear antibody; APAB, antiphospholipid antibody; DLCO, carbon monoxide diffusion capacity; FEV₁, forced expiratory volume in 1 minute; FVC, forced vital capacity; ILD, interstitial lung disease; LFU, lost to follow-up; NA, not available; NSIP, nonspecific interstitial pneumonitis; RF, rheumatoid factor; RNP, ribonucleoprotein; UIP, usual interstitial pneumonitis.
pattern was observed in all cases from transbronchial biopsies, including cases in which the radiographic interpretation was suggestive of UIP. In 5 biopsy specimens, there was extensive parenchymal fibroplasia with honeycomb obliterator changes, compatible with UIP. Two of these cases showed concomitant foci reminiscent of nonspecific interstitial pneumonitis. Eleven biopsy specimens showed evidence of microvascular injury defined by variable luminal and mural fibrin deposition with concomitant interstitial and intra-alveolar hemorrhage. There was also evidence of antecedent microvascular injury delineated by the presence of basement membrane zone thickening and interstitial hemosiderin deposition temporally associated with parenchymal fibroplasia. The chronic vasculopathic changes were more widespread, whereas the more acute changes defined by zones of mural and/or luminal fibrin deposition were focal and temporally associated with foci of organizing pneumonitis.

**Direct Immunofluorescence**

DIF was conducted on native terminal lung parenchyma. Each case had significant deposition of immunoglobulin and complement within the interstitium when observed under oil

**Table 2**

**Summary of Pathology Results**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>NSIP or UIP</th>
<th>Septal Microvascular Injury</th>
<th>Direct Immunofluorescence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NSIP</td>
<td>Yes</td>
<td>IgG(sc/e); IgA(sc); IgM(sc); C1q(sc); C4d; C5b-9(sc/e)</td>
</tr>
<tr>
<td>2</td>
<td>UIP</td>
<td>No</td>
<td>IgG(sc/e); IgM(sc); C1q focal(sc); focal C3; C5b-9(sc); C4d(sc/e)</td>
</tr>
<tr>
<td>3</td>
<td>NSIP</td>
<td>No</td>
<td>IgG(sc/e); IgA(sc); C1q(sc); C3(sc); C4d(sc); C5b-9(sc)</td>
</tr>
<tr>
<td>4</td>
<td>UIP</td>
<td>No</td>
<td>IgG; IgM; 5b-9 nucleolar pattern(sc)</td>
</tr>
<tr>
<td>5</td>
<td>UIP</td>
<td>Yes</td>
<td>C4d; IgG(sc); C1q</td>
</tr>
<tr>
<td>6</td>
<td>UIP</td>
<td>No</td>
<td>IgG(sc); IgA(sc); C1q(sc); C3(sc)</td>
</tr>
<tr>
<td>7</td>
<td>NSIP</td>
<td>Yes</td>
<td>IgG(sc/e); IgM(sc); C1q(sc); C4d focal(sc); C5b-9(sc)</td>
</tr>
<tr>
<td>8</td>
<td>UIP</td>
<td>Yes</td>
<td>IgG(sc/e); IgA(sc/e); C5b-9(e); C1(e); IgM(sc)</td>
</tr>
<tr>
<td>9</td>
<td>NSIP</td>
<td>Yes</td>
<td>IgG(e); IgM(e)</td>
</tr>
<tr>
<td>10</td>
<td>NSIP</td>
<td>Yes</td>
<td>IgG(e); IgM(sc); C1q(sc/e); C3(sc/e); C5b-9(sc/e)</td>
</tr>
<tr>
<td>11</td>
<td>NSIP</td>
<td>No</td>
<td>IgG nucleolar(e); IgA nucleolar(e); C1q nucleolar(e); C3(sc)</td>
</tr>
<tr>
<td>12</td>
<td>NSIP</td>
<td>Yes</td>
<td>C5b-9 3/3(sc); IgA 2/3(e); IgM 3/3(sc)</td>
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<tr>
<td>13</td>
<td>NSIP</td>
<td>Yes</td>
<td>IgG(e); IgA(sc/e); C1q(sc); C3(sc); C5b-9(sc); C3d(sc)</td>
</tr>
<tr>
<td>14</td>
<td>NSIP</td>
<td>Yes</td>
<td>IgG(sc); IgA(sc/e); IgM(sc); C1q(sc); C3(sc/e); C4d(sc/e); C5b-9(sc/e)</td>
</tr>
<tr>
<td>15</td>
<td>NSIP</td>
<td>Yes</td>
<td>IgG and C5b-9 in bronchial wall vessels along with C5b-9 along the bronchial BMZ</td>
</tr>
<tr>
<td>16</td>
<td>NSIP</td>
<td>Yes</td>
<td>IgG(sc); IgA(sc/e); IgM(sc); C1q(sc); C3(sc/e); C4d(sc/e); C5b-9(sc/e)</td>
</tr>
</tbody>
</table>

BMZ, basement membrane zone; e, endothelial cell localization; ND, not done; NSIP, nonspecific interstitial pneumonitis; sc, septal capillary deposition; UIP, usual interstitial pneumonitis.

* Unless otherwise stated, the staining pattern was granular.
immersion. The staining pattern was localized to the interstitium, specifically the septal capillary walls, and/or was observed within the septal capillary endothelium. Of 13 cases, 12 demonstrated granular deposits of IgG within the interalveolar septa, compatible with septal capillary wall localization. Of these, 8 lung biopsy specimens had concomitant IgG deposition localized to the endothelial cells. The deposition pattern was extensive throughout the lung parenchyma and was not specifically localized to areas of parenchymal fibrosis. This pattern was observed in patients with dermatomyositis, anti-Ro–associated SLE, anti-Ro–associated scleroderma, SS, and MCTD. A similar qualitative and quantitative pattern was observed with C1q, C3, C4d, and C5b-9. C5b-9 deposition was most prominent in patients with dermatomyositis. Two cases showed a striking nucleolar staining pattern for IgG within endothelium, one in a case of scleroderma and the other in a case of scleroderma-myositis. Interstitial and endothelial cell granular IgA deposition was seen in patients with RA.

Indirect Immunofluorescence

IIF was done in 6 cases using LMVEC, HUVEC, HEP-2, and rodent lung assays. Indirect assays using pulmonary endothelial cell cytocentrifuged preparations were conducted in 4 patient serum specimens. In 2 patients with dermatomyositis, intense granular nuclear decoration was detected within endothelial cells of human umbilical vein and pulmonary endothelial cells using a fluoresceinated antihuman human IgG antibody. In another patient with dermatomyositis, a weak staining pattern was observed within the LMVEC, whereas none were observed in the HUVEC. In 1 patient with underlying RA, a positive result was obtained using antihuman IgA antibodies, whereas a negative result was obtained using IgG. The neonatal rodent lung assay was conducted in 3 cases. In one case of underlying scleroderma, a striking nucleolar pattern was demonstrated, whereas a second case showed a granular nuclear staining pattern using fluoresceinated human anti-IgA with septal capillary endothelial cell localization. The patient had RA. A third patient with dermatomyositis showed granular deposition for IgG manifesting endothelial cell localization.

Western Blots

Western blot analysis was performed on serum samples from 6 patients; serum samples were not available for the other patients. The serum in each case reacted with multiple bands, however, there were certain consistent antibody responses observed in all cases tested with the antigenic targets manifesting molecular weights of 76 kd, 55 kd, and 40 kd. The bands were intense and characteristically in excess of 10.

Control Samples

DIF Samples

Six cases of other forms of lung inflammation and injury were examined, including 1 of diffuse alveolar injury, 1 of radiation pneumonitis, 2 of chemotherapy-associated pneumonitis, and 2...
of hypersensitivity pneumonitis. Some degree of granular septal deposition of complement and IgM was seen in 4 cases.

**IIF Assays**

In 3 patients with limited scleroderma without pulmonary involvement, the neonatal rodent lung assay was negative. In 4 patients with other types of systemic disease, including hypercholesterolemia, primary APAB syndrome, myelodysplastic syndrome, and bipolar affective disorder, the neonatal rodent lung assay results were negative. In 5 healthy people, it was negative.

The indirect endothelial cell antibody assay using endothelial cell cytocentrifuged preparations was conducted on serum samples from 1 patient with sarcoidosis, 1 with pulmonary hypertension associated with sleep apnea, and 2 patients with primary pulmonary hypertension; results were negative. In samples from 5 healthy control subjects, the assay was negative.

**Western Blot Control Samples**

In samples from 5 patients with emphysema and 5 healthy control subjects, a few bands, ranging from 1 to 4, were observed. Among the more frequently observed bands were those between 45 and 50 kd in 4 of 10 patients and between 70 and 76 kd in 6 of 10 patients.  

**Electron Microscopy**

Electron microscopy was performed in 6 cases. The most striking abnormalities were in the context of septal fibrosis and vasculopathic changes confined to the septal microvasculature.
Discussion

The spectrum of lung disease in the setting of CTD includes lymphocytic interstitial pneumonitis, small vessel vasculitis, rheumatoid noduleus, bronchiolitis obliterans with organizing pneumonitis, bronchocentric granulomatosis, PF, and active chronic lymphocytic and plasmacytic pleuritis.1,13-18 Although PF has been identified in all rheumatologic diseases, it is most prevalent in scleroderma, especially when accompanied by antinucleolar antibodies, dermatomyositis-polymyositis, MCTD, and anti-Ro–associated SLE.4-6,11 Although SS is not typically associated with PF, in the present series, both

Image 8 (Cases 3 and 5) Patient serum antibodies bind to several endothelial proteins. Protein lysates from cultured lung microvascular endothelial cells (LM) and umbilical vein endothelial cells (UV) were separated by polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with patient serum. Bands were detected by chemiluminescence.

Image 9 Western blot result using serum from a healthy control patient. There are a few weak bands; however, the extent of banding qualitatively and quantitatively is much less. Among the more frequently observed bands were those between 45 and 50 kilobases (kb) in 4 of 10 patients and between 70 and 76 kb in 6 of 10 patients. FB, embryonic lung fibroblasts; LM, lung microvascular endothelial cells; SMC, smooth muscle cells; UV, umbilical vein endothelial cells.

Image 10 Activated appearing, swollen capillary endothelial cells with luminal “pseudopodia.” Also note the lamellated basement membrane around the capillary (uranyl acetate-lead citrate, original magnification ×7,000).
patients with SS had antibodies to Ro and/or an antinucleolar staining pattern was observed. PF contributes significantly to the morbidity and mortality in patients with CTD; its pathogenesis in this setting is largely unknown.

In the present study, all patients had light microscopic evidence of active or antecedent septal capillary injury, the hallmarks being those of septal and intra-alveolar fibrin deposition and hemosiderin accumulation typically unaccompanied by significant inflammation. In addition to the light microscopic findings, the ultrastructural findings provided further corroboration of vascular injury. In particular, the constellation of the basement membrane zone abnormalities (ie, thickening, lamellation, collagen deposition, and wrinkling and splitting of the basement membrane zone) are classic ultrastructural findings observed in other forms of immune-based microvascular injury, including the extrapulmonary vascular lesions of lupus erythematosus, dermatomyositis, and scleroderma.19,22

Similar changes of endothelial cell swelling along with more chronic alterations of the basement membrane zone of the microvasculature were described almost 20 years ago in the renal allograft and most recently in the lung allograft, being ascribed to humoral rejection.23 In 1997, Lajoie24 described ultrastructural changes that were attributable exclusively to antibody-mediated rejection whereby the proposed main target of injury was the microvasculature. In this article, a sequence of endothelial cell events was described comprising necrosis of endothelium with subsequent lifting and denuding of the basement membrane that temporally resulted in the complete disappearance of the capillaries with subsequent fibrosis. The ultrastructural changes were attributable exclusively to humoral rejection and essentially mirrored those encountered in our cases.

The presence of vascular injury and its temporal association with fibrosis by light microscopic and ultrastructural studies raises the possibility that an immune-based vascular injury may explain the parenchymal fibrosis. DIF testing exhibited a distinctive pattern of granular deposition within the septal capillary walls and within the endothelium in the biopsy specimens procured from patients in the setting of MCTD, dermatomyositis, SS, and scleroderma, and a nucleolar pattern was observed in patients with scleroderma, SS, and scleroderma/dermatomyositis. The presence of this staining pattern is indicative of a Gell and Coombs type II immune reaction directed against endothelium. All of these patients had antibodies to Ro, RNP, nucleolar-specific antigen, and/or myositis-specific antigens such as OJ and Jo 1. Other studies have shown Ro and RNP localization to endothelium and the association of anti-Ro and anti-RNP antibodies with microvascular disease.25,26

Furthermore, it is well established that the basis of skin and muscle changes in dermatomyositis is related to immune-based microvascular injury with C5b-9 as the effector mechanism.27-29 An oligodot-nucleolar staining pattern localized to endothelium on IIF testing using rodent lung substrate has been correlated with PF.30 Further corroboration of a Gell and Coombs type II immune reaction was the microvascular deposition of C1q and C4d, which are both components of classic complement activation. As expected in cases showing granular endothelial deposition by DIF, the same profile was found with the IIF technique.

Immunoblotting in study group cases demonstrated seroreactivity with multiple proteins derived from endothelial cell lysates. In contradistinction, only a few bands of seroreactivity were seen in the control samples. One would argue that a control sample should have no bands of reactivity; however, that is simply not the case. We have run control samples from several healthy subjects and found reactivity to a few proteins; however, the extent of reactivity qualitatively and quantitatively is much less compared with study group samples. It is well known that the formation of antibodies to neoantigens is occurring all the time in healthy adults; however, there are mechanisms in place that limit the extent of this antibody formation.

Although there were many bands of reactivity in the study group cases, with each case having a relatively unique profile, there were repetitive bands of reactivity observed in all 6 cases: 76, 55, and 40 kd. Possible antigenic targets might include vimentin and tubulin, both of which are approximately 50 kd, and cytokeratin 19, which is reported to be 42 kd. It is interesting to note that although control subjects had fewer bands, reactivity to 1 protein in the 74- to 76-kd range was observed; the specific antigenic target was unclear. The presence of antibodies to a 42-kd protein is significant because cytokeratin 19 is specific to pulmonary endothelium.12 In addition, antibodies to cytokeratin 19 have previously been linked to PF; although it is not certain whether this may simply reflect endothelial injury or a primary response to a pathologic immunogen.10 Earlier studies have shown that the antigenic target implicated in coronary artery transplant disease is vimentin.31 α-Tubulin and vimentin have also been identified as autoantigens of endothelial cells via immunofluorescence and Western blot analysis.32

Other vascular cofactors may be operational in these cases. Half of the cases tested showed positive APABs. Although APABs are typically considered in the context of thrombotic complications, there is increasing awareness of their direct role in endothelial injury. Various studies have demonstrated the ability of APABs to accelerate apoptosis.33,34 Apoptosis is a critical event to epitope spreading by virtue of intracellular antigen displacement, including the extractable nuclear antigens to which many patients in this cohort had antibodies.

Although the link between parenchymal fibroplasia and anti–endothelial cell antibodies in the setting of collagen vascular disease defines a new paradigm, the concept of anti–endothelial cell antibodies in the setting of autoimmune disease, including scleroderma, APAB syndrome, and lupus...
erythematous, is really an old one. The question has always been raised as to whether these antibodies are the basis of the endothelial cell injury observed in the setting of certain CTD syndromes such as dermatomyositis and scleroderma and with chronic graft dysfunction.

Serum from patients with anti–endothelial cell antibodies has been shown to induce cytotoxicity in HUVEC. Up-regulation of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 on cultured human endothelial cells has been observed when endothelial cell cultures have been incubated with anti–endothelial cell antibodies obtained from renal allograft eluates. There is reduced endothelial cell migration in endothelial cell cultures incubated with serum from patients with known circulating anti–endothelial cell antibodies. In addition to these in vitro studies showing a direct effect of these antibodies on endothelial cell function, there are several studies that now establish a link between the presence of anti–endothelial cell antibodies in serum and the severity of disease activity in various autoimmune states.

There is literature on immune-based microvascular injury as a potential basis for parenchymal fibrosis. Wu et al found a significant correlation between the intensity of IIF pulmonary vascular staining using neonatal rat lung as substrate and the degree of PF or hypertension in patients with scleroderma. Other reports describe antibodies to cytokeratins 8, 18, and 19 in patients with IPF; these proteins are uniquely expressed by pulmonary microvascular septal capillary endothelium. Magro et al have also shown microvascular injury along with DIF and IIF features suggesting that immune-based microvascular injury may be an inciting trigger in the evolution of parenchymal fibrosis in patients with IPF.

An interesting and characteristic finding was the presence of IgA anti–endothelial cell antibodies in 3 patients with RA. In a study, antibodies to cytokeratin 18 and epidermal keratin were studied in patients with RA. This study revealed that antibodies of IgA isotype to cytokeratin 18 and epidermal keratin were significantly increased in patients with RA compared with control subjects and with patients with osteoarthritis; they postulated synovial endothelium as a potential source. In a second study, the prevalence of anti–endothelial cell antibodies was assessed in patients with collagen vascular disease. The highest frequency was found in patients with SLE and was primarily of the IgG class. In the RA patient group, only 9.5% had anti–endothelial cell antibodies; however, they were mainly of the IgA isotype. Of interest, in another study, only IgA rheumatoid factor and C3 further increased the probability of histologically proven rheumatoid vasculopathy. One could speculate that anti–endothelial cell antibodies of the IgA isotype may have a role in the propagation of RA-associated PF.

This study described 16 patients with CTD and PF in whom there was serologic and pathologic evidence for an immune-based microvascular injury syndrome. It would seem that there may be multiple antigenic targets based on the heterogeneous pattern observed through our Western blot analysis. Additional confounding vascular factors, primarily in the context of APABs, may also have a role. This is only a pilot study that provides an initial template to explain the basis of PF in the setting of collagen vascular disease. Further studies for additional validation are necessary.

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