Hyperplasia of Type II Pneumocytes in Pulmonary Lymphangioleiomyomatosis

Immunohistochemical and Electron Microscopic Study

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Pulmonary lymphangioleiomyomatosis (LAM), a disease of women, is characterized by (1) proliferation of abnormal smooth muscle cells (LAM cells) in the pulmonary interstitium and along axial lymphatics and lymph nodes of the thorax and abdomen, (2) parenchymal cysts throughout the lungs, and (3) a high incidence of angiomylipomas. The LAM cells differ from normal vascular smooth muscle cells in a number of important respects, including their reactivity for proliferating cell nuclear antigen (PCNA),8 apoptosis-related factors,9 matrix metalloproteinases (MMPs),10,11 estrogen receptors,7,9,12,13 and HMB-45 antibody,14 which is a useful cellular marker for the diagnosis of LAM.

The pulmonary cysts in LAM are distributed widely throughout the parenchyma and have a characteristic appearance on high-resolution computed tomographic study.15,16 Only limited information is available on the morphology of the epithelial cells lining these cysts. Some histologic studies have reported proliferation of type II alveolar epithelial cells in the lining of these cysts,17,18 and other studies have shown that focal micronodular hyperplasia of type II pneumocytes is a rare but distinctive lesion that typically occurs in patients in whom LAM is associated with tuberous sclerosis (see Travis et al13 for review).

During morphologic and immunohistochemical studies of LAM, we noted the consistent presence of numerous type II pneumocytes in the lining of the pulmonary cysts and in noncystic areas of lungs. These morphologic findings correspond to those described as hyperplasia of type II pneumocytes in a large number of conditions, usually in association with acute alveolar injury.19 This term has been used extensively but has not been defined in terms of quantitative criteria, although it has been reported that type II pneumocytes account for 10% of the alveolar cell population in normal lung tissue obtained under ideal conditions of fixation and preparation.20 The diagnosis of hyperplasia of type II alveolar epithelial cells in human lung specimens is usually based on the finding of (1) clusters of these cells, instead of cells occurring singly, and (2) qualitative morphologic alterations, including cuboidal shapes, increased nucleocytoplasmic ratio, enlarged nuclei, prominent nucleoli, and various alterations in their nuclear chromatin.21,22

To assess the occurrence of hyperplasia of type II pneumocytes lining the parenchymal cysts in lymphangioleiomyomatosis (LAM).

Methods.—Immunohistochemical and electron microscopic studies were performed on lung tissue from 22 women with pulmonary LAM.

Results.—Epithelial cells that reacted with PE-10 (a mouse monoclonal antibody that recognizes the surfactant apoprotein A in type II pneumocytes) and TTF-1 (an antibody that identifies nuclear transcription factor found in type II pneumocytes) were the predominant cell type lining the surfaces of lesions of LAM and normal areas of lung. Scanning and transmission electron microscopic studies confirmed that these cells were type II pneumocytes as demonstrated by (1) apical microvilli, (2) electron-dense lamellar bodies, and (3) cytoplasmic projections that extended from the basal surfaces into the underlying connective tissue, where they made extensive contact with interstitial connective tissue cells. A few cells had morphologic characteristics of type I pneumocytes, including large flat surfaces lacking microvilli. Cells that appeared intermediate between type I and type II pneumocytes were observed occasionally.

Conclusions.—These observations and the reactivity of these cells with antibody to proliferating cell nuclear antigen demonstrate that extensive hyperplasia of type II pneumocytes is a major characteristic of LAM.
mocytes in LAM, the present study was undertaken using methods of histology, immunohistochemistry, confocal microscopy, and scanning and transmission electron microscopy.

**MATERIALS AND METHODS**

**Patients Studied**

The study group consisted of 22 women (age range, 26–55 years; mean ± SD age, 37.6 ± 8.2 years) in whom the diagnosis of LAM was made on the basis of clinical, pulmonary function, high-resolution computed tomography, and histologic studies. In all cases, the diagnosis was confirmed by histochemical demonstration of the reactivity of the LAM cells with HMB-45 antibody. Lung tissue was obtained from these patients by open biopsy at the time of their initial clinical evaluation (9 patients) or at autopsy (2 patients) or pulmonary transplantation (11 patients).

The mean duration of the illness, estimated from the onset of symptoms to the time when the diagnosis was established, was 6 months (range, 1–24 months) for the patients studied by biopsy versus 70 months (range, 7–264 months) in patients studied at either necropsy or transplantation. However, the hyperplasia of type II pneumocytes was not significantly different in the 2 groups of patients, and for this reason the data for all patients are presented together. For control purposes, histologic and immunohistochemical studies were made to evaluate the distribution of type II pneumocytes in morphologically normal lung tissue from 4 patients who underwent lobectomy for solitary pulmonary nodules. The study was approved by the Intramural Review Board of the National Heart, Lung and Blood Institute.

**Histologic Study**

For histologic study, tissues were fixed with 10% formalin, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin-eosin and the Masson trichrome and Movat pentachrome methods.11

**Immunohistochemical Staining**

Single and dual immunohistochemical staining methods with paraffin sections of formalin-fixed tissue were used. The immunoperoxidase (Envision System, Dako, Carpinteria, Calif) was used for single staining. The dual immunofluorescence method was used for demonstrating immunoreactivity for various combinations of a mouse monoclonal antibody and a rabbit polyclonal antibody, as indicated below. Mouse monoclonal antibodies directed against the following components were used: HMB-45, which recognizes gp10023 and serves as a marker for epithelioid LAM cells. Many of the epithelial cells in a peribroncholar LAM nodule demonstrate a positive cytoplasmic reaction for PE-10 (C), with no staining in the other bronchiolar epithelial cells (arrow). A positive reaction for TTF-1 is observed in the nuclei of many epithelial cells covering the nodules of LAM cells (D) but not in macrophages (arrow). Numerous positive epithelial cells are also present in areas not involved by LAM (E) (C and D, original magnification ×200; E, original magnification ×400). (F) Reaction for proliferating cell nuclear antigen. Peroxidase method and nuclear counterstaining with hematoxylin. Many of the LAM cells show a positive nuclear reaction. Some of the epithelial cells covering the LAM nodule also are positive (arrows) (original magnification ×400).

**Figure 1.** Histopathologic and immunohistochemical findings in pulmonary lymphangioleiomyomatosis (LAM). (A) Low-magnification view showing nodules of LAM cells (hematoxylin-eosin, original magnification ×120). (B) High-magnification view of the section shown in A. The epithelial cells covering the LAM nodule are numerous and plump, with relatively large nuclei (1-μm-thick section of tissue embedded in epoxy resin, alkaline toluidine blue stain, original magnification ×400). (C, D, and E) Reaction for PE-10 (C) and TTF-1 (D and E) (peroxidase method and nuclear counterstaining with hematoxylin). Many of the epithelial cells in a peribroncholar LAM nodule demonstrate a positive cytoplasmic reaction for PE-10 (C), with no staining in the other bronchiolar epithelial cells (arrow). A positive reaction for TTF-1 is observed in the nuclei of many epithelial cells covering the nodules of LAM cells (D) but not in macrophages (arrow). Numerous positive epithelial cells are also present in areas not involved by LAM (E) (C and D, original magnification ×200; E, original magnification ×400). (F) Reaction for proliferating cell nuclear antigen. Peroxidase method and nuclear counterstaining with hematoxylin. Many of the LAM cells show a positive nuclear reaction. Some of the epithelial cells covering the LAM nodule also are positive (arrows) (original magnification ×400).
Figure 2. Confocal images of sections of lung after dual labeling. Sections were labeled for PE-10, shown by red fluorescence in all panels, plus either matrix metalloproteinase 2 (MMP-2) (A, B, and C) or membrane-type matrix metalloproteinase 1 (MT-1-MMP) (D), which are indicated by green fluorescence. Yellow fluorescence shows either colocalization of the red and green signals or autofluorescence. Nuclei show blue fluorescence due to counterstaining with 4′,6′-diamidino-2-phenylindole. (A, B, and C) Reactivity for PE-10 and MMP-2 in a lymphangioleiomyomatosis (LAM) lesion (A) and non-LAM area (B) and normal lung (C). The LAM cells show a strong reaction for MMP-2, and those cells located near the surfaces of the nodule are in close apposition to the overlying PE-10-positive cells. Colocalization of PE-10 and MMP-2 is observed in many epithelial cells and macrophages (B). In normal lung tissue (C), PE-10-positive cells are sparsely distributed in the alveolar walls (A and B, original magnification x400; C, original magnification x250). (D) Reactivity for PE-10 and MT-1-MMP in LAM lesion. Epithelial cells show colocalization of PE-10 and MT-1-MMP. Yellow autofluorescence of connective tissue is evident in the thickened wall of a cyst (original magnification x630).

as red (Vector Laboratories, Burlingame, Calif; dilution, 1:100). The antibodies against MMP-2 (dilution, 1:200), MT-1-MMP (dilution, 1:100), and PRs (dilution, 1:10) were reacted with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Vector; dilution, 1:100). The sections were counterstained for 15 minutes with an aqueous 0.01% solution of 4′,6′-diamidino-2-phenylindole for identification of nuclei. The preparations were then examined by confocal microscopy. Details of the staining and confocal microscopy procedures have been previously described.11 These antibodies were used to evaluate possible relationships between the hyperplasia of type II pneumocytes and the localization of specific subtypes (spindle shaped and epithelioid) of LAM cells, which can be identified as described in detail in previous publications from this laboratory.10,11 The other antibodies (TTF-1 and PE-10) provided 2 independent means for the immunohistochemical identification of type II pneumocytes.

Immunohistochemical control procedures used in conjunction with all the methods described herein consisted of (1) omission of the primary antibody from the staining protocol and (2) replacement of the primary antibody by an equivalent amount of normal IgG from the same animal species. Both these control procedures gave negative results in all instances.

**Scanning Electron Microscopy**

For scanning electron microscopic study, tissues from 5 patients were fixed with 3% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.2, dehydrated with a graded series of ethanol and propylene oxide, and embedded in PolyBed 812. Thick sections (1 µm) were stained with alkaline toluidine blue for light microscopic examination and selection of areas for electron microscopic study. Ultrathin sections of the selected areas were stained with lead citrate and uranyl acetate and examined with a transmission electron microscope (JEOL 1200 EX).

**RESULTS**

**Histopathologic Observations**

The histopathologic appearance of the lung tissue was characterized by proliferation of LAM cells, which formed nodules of various sizes, and by formation of cysts of various sizes (Figure 1, A). The pneumocytes and the walls of the cysts covering the LAM nodules were plump, with relatively large nuclei (Figure 1, B). As confirmed by the immunohistochemical and electron microscopic studies described below, these cells were hyperplastic type II pneumocytes. The number of these cells was counted in 5 randomly selected microscopic fields, at a magnification of 250, in each of the patients. The frequency of occurrence of such cells was much greater than that observed in normal alveolar surfaces, in which they are found singly or in pairs, rather than as the predominant cell population.

**Immunoperoxidase Staining**

Most of the epithelial cells covering the nodules of LAM cells and the walls of the cysts gave a positive cytoplasmic reaction with PE-10 and a positive nuclear reaction with TTF-1 (Figure 1, C and D). Alveolar macrophages gave a moderate-to-strong reaction with PE-10 but a negative re-
Figure 3. Scanning electron micrographs of lung tissue from patients with lymphangioleiomyomatosis (LAM). (A) Low-magnification view shows numerous round-shaped cells covering the surface of LAM nodules. The cut surface of a LAM nodule is seen at lower right (original magnification ×500). (B) Higher-magnification view of a relatively normal area, showing a small alveolar space lined by a mixture of type II pneumocytes with surface microvilli and type I pneumocytes with round, flat surfaces (original magnification ×1600). (C) Low-magnification view shows thin-walled cysts of various sizes and a honeycomb-like appearance (original magnification ×100). (D) Higher-magnification view of a cyst wall reveals an extensive proliferation of type II pneumocytes that possess numerous microvilli (original magnification ×3500). (E) Surface of a cyst, showing many rounded cells along with a few flattened cells; pores of Kohn are visible in the lower left area (original magnification ×800). (F) High-magnification view of a cyst wall with numerous rounded cells with and without microvilli, along with a few flattened cells that have no microvilli (original magnification ×2200).

action with TTF-1 (Figure 1, D). No other cells or extracellular components reacted with PE-10 or TTF-1.

Areas of normal architecture, in which the pulmonary parenchyma was not involved by either cyst formation or infiltration of LAM cells, were evident in 18 patients. PE-10– and TTF-1–positive cells were markedly increased in numbers in these areas (Figure 1, E). Confirmation that such areas were free of infiltrates of LAM cells was obtained by detailed examination of consecutive sections, ranging from 20 to 30, that were cut from each tissue block and used for different staining procedures that served to identify type II pneumocytes and LAM cells.

The overall numbers of PE-10–positive cells were counted in 5 randomly counted fields at a magnification of 250 in each patient. The mean (SD) frequency of these cells was 45.2 ± 14.5 in the entire group of patients, and the count was significantly higher than in normal lung tissues (9.3 ± 2.6; P < .001). The immunohistochemical reactivity observed for PCNA and various markers of LAM cells was similar to that found in previous studies.8–11,13 The type I pneumocytes were unreactive for all these components. Approximately 5% of the type II pneumocytes gave a positive reaction for PCNA (Figure 1, F).

Confocal Microscopic Study

Confocal microscopic studies were made of preparations stained by dual labeling methods for the demonstration of PE-10 antibody and various LAM cell markers. The results showed that the LAM cells located near the surfaces of the nodules often were in close apposition to the overlying type II pneumocytes (Figure 2, A and D). PE-10–positive cells were more numerous, even in areas in which LAM cells were few or absent, than in normal control tissue (Figure 2, B and C). Thus, study of preparations after dual staining for type II pneumocytes and LAM cells showed that the foci of hyperplasia of type II pneumocytes were more widespread than those of infiltration of LAM cells.

Scanning Electron Microscopic Findings

Scanning electron microscopic studies were made to evaluate the surface morphologic structure of the alveoli and the cystic spaces. The degree of cystic dilation of the air spaces ranged from minimal in 1 patient (Figure 3, A) to marked in 4 patients (Figure 3, B through F). In all cases, cells with round shapes and apical microvilli, corresponding to type II pneumocytes, were predominant in both non-LAM areas (Figure 3, B) and on the surfaces of cysts (Figure 3, D). This finding was in accord with the histologic and immunohistochemical observations described herein. Other cells lining the cystic spaces corresponded to type I pneumocytes, as shown by their larger surface areas, apical surfaces, and absence of microvilli (Figure 3, A). Although many of the round cells corresponding to type II pneumocytes had abundant microvilli, numerous other round epithelial cells contained only a few microvilli, suggesting the possibility that they were morphologically intermediate between type I and type II.
pneumocytes (Figure 3, A, E, and F). Structures resembling enlarged pores of Kohn were occasionally evident in the walls of the cystic spaces (Figure 3, E). Ciliated cells were not clearly evident in the cystic spaces.

**Transmission Electron Microscopic Findings**

Transmission electron microscopic observations confirmed that many of the cells lining the surfaces of the pulmonary cysts and the more normal alveolar spaces in all 6 patients with LAM were type II pneumocytes (Figure 4, A). Most of these cells were cuboidal in shape; however, other cells were more flattened and elongated. The luminal surfaces of typical type II pneumocytes had numerous short microvilli. The membranes along the lateral surfaces formed complex, interdigitating junctions with those of adjacent epithelial cells. Highly electron-dense cytoplasmic lamellar bodies of the type well known to be present in type II pneumocytes were the most distinctive feature of these cells. The basal surfaces of the type II pneumocytes were invested by a well-defined basement membrane through which small cytoplasmic projections penetrated into the underlying connective tissue space (Figure 4, B). Some of these projections made direct contacts with cytoplasmic processes of connective tissue cells in LAM nodules. These connections consisted of close appositions between nonspecialized areas of the plasma membranes of the 2 cells.

We also observed surface-lining cells that possessed few or no lamellar bodies and/or surface microvilli (Figure 5, A). Such cells were considered to be suggestive of a transition from type II to type I pneumocytes. The latter were much less frequent than type II pneumocytes and were characterized by very flat shapes and a thin cytoplasm. These type I pneumocytes did not have microvilli, lamellar bodies, or cytoplasmic projections along their basal surfaces (Figure 5, B).

**COMMENT**

The present study demonstrates the occurrence of hyperplasia of type II pneumocytes in LAM not only in the parenchymal cysts but also in areas of nearly normal alveolar architecture (as determined by immunohistochemical staining procedures showing few or no LAM cell infiltrates). Evidence of this hyperplasia was derived from the following 4 types of independent observations: (1) histologic study, which revealed numerous type II pneumocytes; (2) immunohistochemical staining demonstrating the reactivity of these cells with PE-10 and TTF-1 antibodies; (3) scanning electron microscopy, which disclosed sur-
face features typical of type II pneumocytes; and (4) transmission electron microscopy, which provided final confirmation of the identification of these cells.

PE-10 antibody has been considered to be specific for type II pneumocytes, although Clara cells have been reported to show some reactivity because they can also express surfactant apoprotein A. However, the morphologic structure typical of Clara cells (flame-shaped luminal projections that are smooth surfaced and lack microvilli) was not observed in these hyperplastic epithelial cells. We also found reactivity for PE-10 antibody in alveolar macrophages, presumably because these cells can internalize surfactant secreted by other cell types into alveolar lumina. We also used TTF-1 antibody for the identification of type II pneumocytes. The usefulness of this antibody for this purpose has been demonstrated.

Some of the type II pneumocytes observed in the present study were flattened and elongated, and for this reason they were difficult to distinguish from type I pneumocytes in histologic preparations. Transmission and scanning electron microscopic study showed that the hyperplastic epithelial cells corresponded to type II pneumocytes. Direct contacts between type II pneumocytes and connective tissue cells of the alveolar walls have been observed in normal lung and in a variety of fibrotic lung disorders. However, the significance of these connections remains to be determined. The observation of cells that appear morphologically intermediate between type I and type II pneumocytes is consistent with the concept that the latter cells are precursors of type I pneumocytes. However, morphologic aspects of this transition have received very little attention.

Hyperplasia of type II pneumocytes develops as a response to alveolar injury in a variety of pulmonary disorders, especially in the setting of inflammation and interstitial fibrosis and during the healing phase of diffuse alveolar damage. Nevertheless, in our patients these interstitial alterations were not present in areas away from LAM infiltrates. Proliferation of type II pneumocytes has been reported in emphysema. This proliferation may be prominent during the early stages of healing of the elastase-induced pulmonary damage. However, advanced emphysematous lesions usually have smooth surfaces, in contrast to the numerous surface microvilli found in the present study.

The pulmonary cystic spaces (honeycombing) in patients with advanced stages of various fibrotic lung disorders also differ from those observed in the present study, since they are lined not only by type II pneumocytes but also by cells presumed to be of bronchiolar origin. Taken together, these observations suggest that the hyperplasia of type II pneumocytes in LAM may represent a diffuse response to mitogenic stimuli rather than to focal alveolar injury. The complexity of this problem is emphasized by recent observations showing that hyperplasia of type II pneumocytes can be induced by a wide variety of growth factors, including granulocyte-macrophage colony-stimulating factor, peptidyl-glycine α-amidating mono-oxygenase, keratinocyte growth factor, transforming growth factor β1, and insulin-like growth factor 1.

In summary, the present study shows that the epithelial cells lining both the cystic spaces and the remaining, more nearly normal alveoli in patients with LAM consist mainly of proliferating type II pneumocytes, as determined by immunohistochemical staining and by scanning and transmission electron microscopic studies.

References
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