—Review—

Lymphangioleiomyomatosis (LAM)

A Review of Clinical and Morphological Features

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Abstract

A review is presented of the clinical and morphological manifestations of lymphangioleiomyomatosis (LAM), a systemic disorder of unknown etiology that affects women. The clinical features include dyspnea, hemoptysis, recurrent pneumothorax, chylothorax, and chylous ascites. It is characterized by: 1) proliferation of abnormal smooth muscle cells (LAM cells) in pulmonary interstitium and along the axial lymphatics of the thorax and abdomen; 2) thin-walled pulmonary cysts, and 3) a high incidence of angiomyolipomas. The pulmonary cystic lesions have a characteristic appearance on high resolution computed tomography. The most specific method for diagnosing LAM is lung biopsy to demonstrate the presence of LAM cells, either by their characteristic histological appearance or by specific immunostaining with HMB-45 antibody. LAM cells differ in several important respects from the types of smooth muscle cells normally present in lung. Their reactivity with HMB-45 antibody is localized in stage I and stage II melanosomes. LAM cells show additional evidence of incomplete melanogenesis, and the significance of these observations remains to be determined. Two types of LAM cells are recognized: 1) small, spindle-shaped cells that are centrally located in the LAM nodules and are highly immunoreactive for matrix metalloproteinase-2 (MMP-2), its activating enzyme (MT-1-MMP), and proliferating cell nuclear antigen (PCNA), and 2) large, epithelioid cells that are distributed along the periphery of the nodules and show a high degree of immunoreactivity with HMB-45 antibody and with antibodies against estrogen and progesterone receptors. Types of treatment used for LAM include oophorectomy, administration of Lupron or progesterone and in very severe cases, pulmonary transplantation (following the onset of respiratory insufficiency, not relieved by O_2). (J Nippon Med Sch 2000; 67: 311—329)

Key words: lymphangioleiomyomatosis, lung, radiology, pathology, histochemistry

Introduction

Lymphangioleiomyomatosis (LAM) is a systemic disorder that affects predominantly women during their reproductive years[1,2]. It is characterized by: 1) proliferation of abnormal smooth muscle cells, usually designated as LAM cells, in pulmonary interstitium

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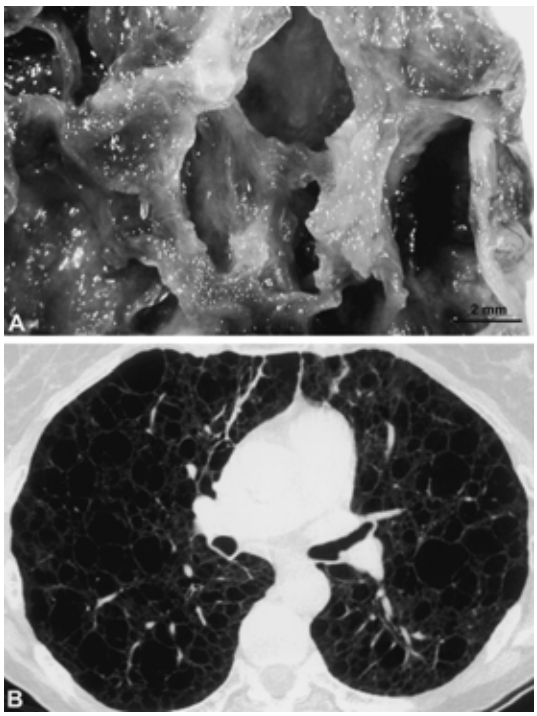


Fig. 1 Anatomical and Radiological features of LAM. **A**: Dissecting microscope view of cut surface of lung removed from a patient with LAM at the time of pulmonary transplantation, showing numerous thin-walled cysts of various sizes. **B**: Nonenhanced axial 1 mm high resolution CT section of the chest of a 53 year old patient with LAM. Diffuse, thin-walled pulmonary cysts of varying sizes are shown. Compare with **Fig. 1 A**.

and along the axial lymphatics of the thorax and abdomen; 2) formation of thin-walled cysts (**Fig. 1 A**) that are distributed diffusely throughout the lungs, and 3) a high incidence of angiomyolipomas[3,4]. The gross anatomical, histological and radiological features of these lesions are illustrated in **Figs. 1 A, 1 B, and 2 A to 2 F**. The LAM cells are thought to be derived from perivascular cells that are located along the walls of lymphatic vessels (see Travis et al.[5] for review). The disease is most often sporadic. However, it also has been reported to occur in about 3% of female patients with tuberous sclerosis complex (TSC)[5,6], a genetically transmitted disorder resulting from mutations in one of two genes, TSC 1 or TSC 2[7]. It appears that female patients with TSC have a significant incidence of pneumothorax and cystic pulmonary lesions[6], and this association is more frequent than was previously believed, as suggested by a recent study[6] that demonstrated a high incidence of spontaneous pneumothorax and cystic pulmonary lesions in patients who had tuberous sclerosis but had no pulmonary symptoms.

Clinical Findings

The disease is characterized clinically by dyspnea, hemoptysis, recurrent pneumothorax, evidence of chylothorax, chylous ascites or chylopericardium, and abdominal masses, which may represent either massively enlarged lymph nodes or angiomyolipomas[8-11]. The abdominal masses may be asymptomatic or may cause non-specific symptoms. In some patients they are the initial manifestation of LAM, and investigation of such lesions can lead to the previously unsuspected diagnosis of pulmonary LAM[12].

In a series of 69 patients examined by Urban et al[11], LAM was diagnosed after menopause in about 10% of cases. LAM was first recognized during pregnancy in 20% of cases, and clear exacerbation of LAM was observed during pregnancy in 14% of cases. In this series of patients, chylothorax was observed in 29% of patients during the overall course of the disease with recurrence in 6; chylous ascites was diagnosed in 12% of patients. The abdominal masses observed included angiomyolipomas, which were found in 32% of the patients, abdominal lymphadenopathy, found in 24%, and uterine leiomyomas, which occurred in 41% of the patients.

Bronchoalveolar Lavage

Chu et al.[10] reported the results of bronchoalveolar lavage (BAL) in six normal female volunteers (age, 38.2 ± 10.8 years) and 16 LAM patients (39.4 ± 7.2 years) with $FEV_1 \geq 45\%$ of predicted value and $PaO_2 \geq 65$ mmHg. The total amounts of cells recovered per BAL, the number of cells per milliliter of lavage fluid, and the numbers of cells per microliter of epithelial lining fluid (ELF) were similar in both groups. The percentage of lavage fluid recovered was significantly lower, and the relative amounts of ELF in lavage fluid were significantly higher in LAM patients. The differential cell counts in BAL fluid were similar in LAM patients and control subjects. Pigment-laden macrophages were present in BAL fluid in 13 LAM patients and 2 normal subjects. The percentage of pigment-

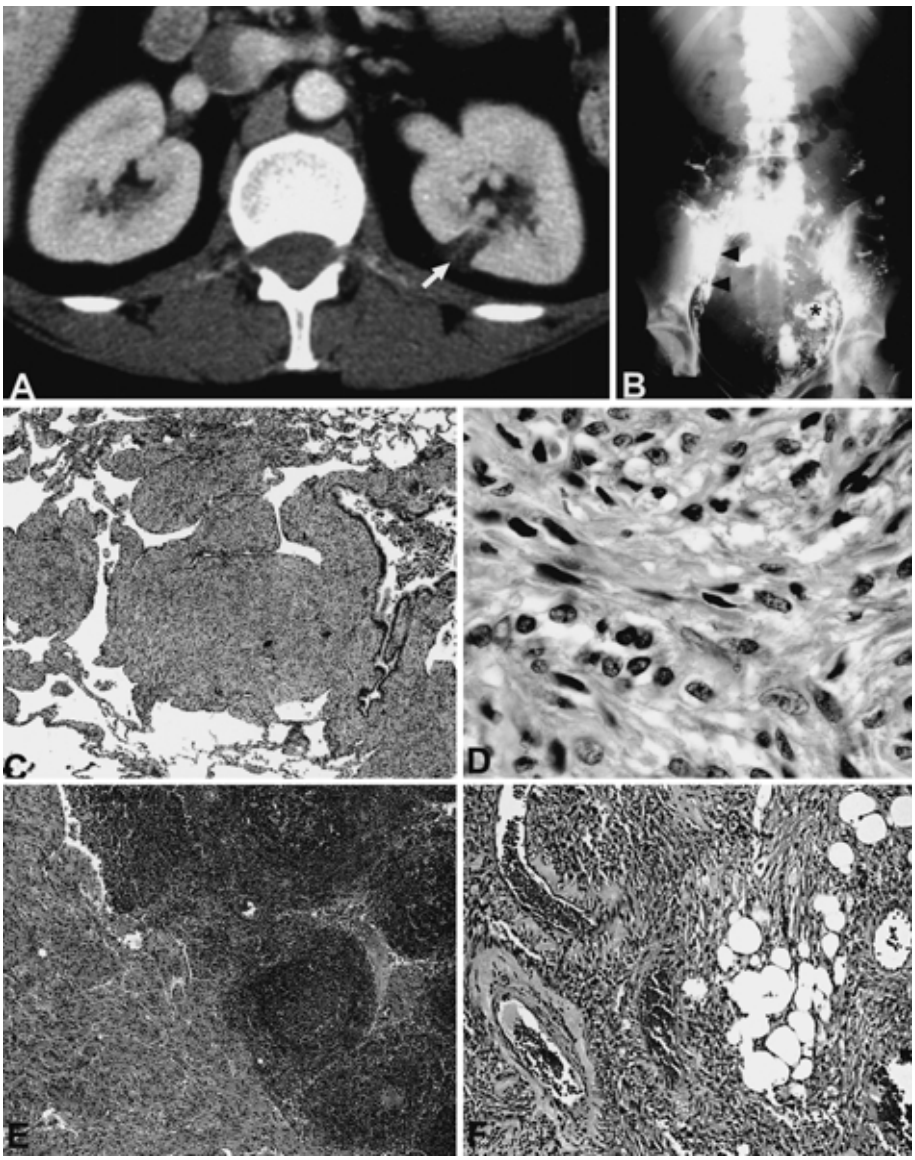


Fig. 2 Radiology and histopathology of LAM lesions. **A**: Enhanced axial 10 mm CT section of the abdomen of a 32 year old patient shows a left renal fatty mass (arrow) consistent with angiomyolipoma. **B**: Lymphangiogram of patient with extrapulmonary LAM involving large areas of retroperiteneum. A plexus of dilated lymphatic vessels (arrowheads), with marked stasis and pooling (asterisk), is evident in the pelvis. **C**: Low magnification view of section of lung, showing nodules of LAM cells and relatively thick-walled cysts. (H & E stain, ×50). **D**: Lung biopsy specimen showing the morphological heterogeneity of LAM cells: small to medium-sized spindle-shaped cells and large epithelioid cells. (H & E stain, ×1,000). **E**: Histology of extrapulmonary LAM. Retroperitoneal mass consists of fascicles of proliferating LAM cells separated by narrow lymphatic channels. Note aggregates of lymphoid cells forming follicular centers. (H & E stain, ×100). **F**: Section of angiomyolipoma shows numerous adipose tissue cells and scattered LAM cells. (H & E stain, ×100).

laden macrophages in LAM patients was significantly higher than in normal subjects.

Radiological Findings

The pulmonary cysts in LAM are rarely evident on routine chest radiographs. Only 8% of the patients have cysts shown by radiographs, whereas 100% have cysts shown by high resolution chest tomography (HRCT)[13]. On HRCTs, the cysts appear to be distributed homogeneously in both lungs (**Fig. 1 B**). In a few patients, the cysts are slightly less numerous in

the upper than in the lower lung zones. The extent of pulmonary cysts varies widely among the patients. The size of the cysts may range from a few millimeters to several centimeters[10].

Other abnormalities on CT of the chest included retrocrural adenopathy in nine patients, pleural effusion in five, dilated thoracic duct in four, pericardial effusion in two, and pneumothorax in two. Among five patients with pleural effusions, four had chylous effusions and one had an effusion that was too small for thoracentesis. In 22 patients, CT and ultrasonography revealed solid renal masses. Nine of the the patients had multiple masses and 6 had bilateral masses. A total of 51 solid renal masses were observed, 34 in the left kidney and 17 in the right. The average size of these masses was 14 ± 1.6 cm. The angiomyolipomas had a characteristic appearance on HRCT of the abdomen (**Fig. 2 A**) and were hyperechoic on ultrasonographic study. Retroperitoneal adenopathy was found in 27 of the patients studied by Chu et al[10]. Fat attenuation in retroperitoneal lymph nodes was observed in 8 patients. Pelvic adenopathy was observed in 4 patients. Lymphangiography (**Fig. 2 B**) was found to be useful to demonstrate retroperitoneal involvement by LAM. Three patients had other findings, including unilateral, single renal cysts in 3 patients. All renal cysts were ≤1 cm in diameter.

In a recent study by Avila et al[14], 80 patients with LAM underwent chest and abdominopelvic CT and ultrasonography. The abdominal findings were compared with the severity of pulmonary disease in HRCT. Sixty-one (76%) of the 80 patients had positive abdominal findings, including renal angiomyolipomas in 43 patients (54%), enlarged abdominal lymph nodes in 31 (39%), and lymphangiomyoma in 13 (16%). Less common abnormalities included ascites in 8 (10%), dilation of the thoracic duct in 7 (9%), and hepatic angiomyolipomas in 3 (4%). There was a significant correlation between enlarged abdominal lymph nodes and greater severity of the lung disease.

Pulmonary Function Studies

Three aspects of pulmonary function have been the subject of special attention in LAM: the rate of decline of FEV_1, the decrease in DLCO, the obstruction of airflow and its response to bronchodilators[15,16]. Lazor et al.[17] examined serial FEV_1 measurements in 50 cases of LAM with a mean follow-up of 5 ± 4 years. The mean rate of FEV_1 decline, determined by linear regression, was −115 ± 176 m*l*/year. To evaluate predictive factors of rapid deterioration, they compared the 2 extremes of the study population, respectively defined by rates of FEV_1 decline ≤ 25th percentile and ≥ 75th percentile. The author concluded that there was no significant difference between the 2 groups in symptoms and signs, use of oral contraceptives, pregnancy, menopause, angiomyolipoma, pneumothorax, chylothorax, chylous ascites, initial FEV_1, results of imaging, or hormonal treatment. The data suggested that the absence of uterine leiomyomas, lower initial DLCO, and lower initial $PaCO_2$, were associated with a more rapid rate of FEV_1 decline in pulmonary LAM.

Taveira-Da Silva et al.[18] found reversible obstruction of airflow in 25% of 143 patients with LAM. A response to bronchodilators was associated with a higher proportion of LAM cells in lung biopsy specimens and with a greater decline of airflow. Inflammation of the airways was found in 61% of 74 lung specimens examined, but did not correlate with obstruction to airflow. The latter, whether fixed or reversible, did not correlate with the severity of the disease. However, DLCO was a useful predictor. Taveira-Da Silva et al.[18] concluded that a positive response to bronchodilators was found in patients with accelerated loss of lung function, and that the degree of impairment of DLCO correlated with the severity of the disease.

To assess the correlations between the results of pulmonary function tests and ventilation-perfusion scintigrams, CT scans, and chest radiographs, Avila et al.[13] studied 39 patients with LAM. Imaging abnormalities were found on 92% of perfusion scintigrams, 79% of chest radiographs, 100% of CT scans, and 100 % of HRCT. On ventilation scintigrams, 28 (72%) of the patients demonstrated a speckling pattern. On CT scans, all patients had pulmonary cysts. The extent of disease on chest radiographs and CT scans, cyst size, ventilation-perfusion abnormalities, and degree of speckling were inversely correlated with FEV_1, and the ratio of FEV_1 to forced vital capacity (FVC) but not with FVC and total lung capacity.

Differential Diagnosis

LAM must be distinguished from other pulmonary disorders associated with proliferation of smooth muscle cells or formation of parenchymal cysts, or both[5]. As discussed in detail by Travis et al.[5], these disorders are most commonly distinguished from LAM on the basis of HRCT and histologic study of pulmonary tissue. Diseases to be considered in the differential diagnosis of proliferation of pulmonary smooth muscle cells include idiopathic pulmonary fibrosis, chronic stages of hypersensitivity pneumonitis, primary pulmonary leiomyomata, benign metastasizing leiomyomata and pulmonary leiomyosarcomas. In most instances, the correct diagnosis can be established by histologic study; however, specific immunostaining of LAM cells with HMB-45 antibody (see below) can be very useful in the evaluation of these problems, since the smooth muscle cells in other pulmonary disorders are unreactive with this antibody[19].

Various cystic disorders of the lung can be easily distinguished from LAM on the basis of their appearance on HRCT imaging[14]. In this category, the most important entity to exclude is pulmonary Langerhans' cell granulomatosis (pulmonary histiocytosis X), which is associated with recurrent pneumothorax and with formation of parenchymal cysts. The diagnosis of this disorder can be established by the demonstration of increased numbers of Langerhans' cells in BAL fluid and/or by the specific identification of these cells (either by immunohistochemical staining with OT-10 antibody or by the ultrastructural demonstration of their specific Birbeck granules)[20,21]. Langerhans' cells do not react with HMB-45 antibody. In addition, in PLCG, the HRCT shows nodules as well as cysts with more involvement of the upper lobes and sparing of the costophrenic angles.

Gross Anatomic Findings

Gross anatomic findings in the lungs are distinctive, with diffuse, bilateral cystic changes throughout. The lungs are enlarged, as in severe emphysema. Most cysts are 0.5-2 cm in size, but in some cases they can measure over 10 cm. The diffuse nature of the cystic changes in pulmonary LAM can be best appreciated by inspection of the cut surfaces of the lungs, using a dissecting microscope and incident light illumination (**Fig. 1 A**).

Histologic Findings

Pulmonary LAM: In the early stages of the disease, the infiltrates of LAM cells may be overlooked and the biopsy misinterpreted as showing either emphysema or normal lung. The LAM cells are typically found in small clusters or nests at the edges of the cysts and along pulmonary blood vessels, lymphatics and bronchioles (**Fig. 2 C**). The infiltration of LAM cells in the walls of vessels and distal airways can lead to vascular destruction and foci of hemosiderosis; bronchiolar obliteration also may occur. The proliferating LAM cells grow in nodular or haphazard arrangements, unlike the orderly, concentric or parallel patterns of normal smooth muscle cells in the airways and blood vessels. Mitotic figures and cytologically atypical cells are rare. A definitive diagnosis of LAM is made on the basis of the identification of LAM cells in lung biopsy specimens. The LAM cells are morphologically heterogeneous[5] and can be classified into two major types: 1) smaller, spindle-shaped cells (which may appear rounded or oval depending upon the plane of sectioning), and 2) larger, epithelioid LAM cells with more abundant cytoplasm (**Fig. 2 D**). These two types of LAM cells have different distributions in the LAM nodules, in which the smaller, spindle-shaped cells are centrally located, whereas the epithelioid cells tend to occur in peripheral regions of the nodules. These observations and the immunohistochemical studies described below have led to the concept that the LAM cells replicate in the central regions of the nodules and undergo progressive growth and differentiation as they migrate to the periphery of the nodules[22]. In the late stages of the disease, the LAM cells are more irregularly arranged, particularly along the walls of the cysts, and are admixed with variable amounts of connective tissue[22].

The pulmonary parenchymal cysts in LAM are characterized by progressive dilatation of the air spaces, and this alteration is associated with hyperplasia of type II pneumocytes[23] and destructive changes

in the elastic fibers and collagen in the walls of the cysts, which undergo progressive thinning[23]. Proteolytic enzymes released by the LAM cells are thought to play a critical role in the formation of these cysts. Recent studies have indicated that matrix metalloproteinases (MMPs) may be responsible for at least part of this damage[24]. It has been suggested that an imbalance in the α-1-antitrypsin system also may play a role in the proteolytic damage, but data to support this concept are lacking[25].

The hyperplasia of type II pneumocytes can be found in areas that do not show accumulations of LAM cells[23]. In contrast to these changes, focal micronodular hyperplasia of type II pneumocytes has been observed as a distinctive lesion in patients with tuberous sclerosis[5] with or without LAM. These lesions consist of ill-defined nodular masses composed of alveoli lined by numerous type II pneumocytes. These nodules often are adjacent to focal areas of emphysematous change, and it has been suggested that they induce obstruction to airflow[5].

Extrapulmonary LAM: Matsui et al.[26] described the lesions of extrapulmonary LAM that affected lymph nodes of the mediastinum and retroperitoneum in 22 cases in which histopathologic study was necessary to establish the diagnosis. In a majority of these patients, the diagnosis of extrapulmonary LAM preceded that of pulmonary LAM, usually by 1 to 2 years. Eleven patients had distinct symptoms, including chylous pleural effusion and/or ascites, abdominal pain and other palpable masses. In the other 11 patients, the masses caused no symptoms. Well-circumscribed, encapsulated masses, measuring up to 20 cm in size, occurred in the mediastinum in two patients, the upper retroperitoneum in 15, extensive areas of the retroperitoneum in 2, and the pelvis in 3. Masses exceeding 3 cm in diameter contained large, multiple cysts filled with yellow-tan chylous fluid. Histologically, the masses were characterized by a proliferation of LAM cells arranged in fascicular (**Fig. 2 E**), trabecular, and papillary patterns, which were associated with slit-like vascular channels. In several cases, the proliferation of LAM cells extended beyond the connective tissue capsule of the extrapulmonary mass. The LAM cells varied from small, spindle-shaped cells to large epithelioid cells. Immunohistochemical studies showed strong reactivity of most LAM cells for α-smooth muscle actin and smooth muscle myosin heavy chains and a weak to moderate reactivity of a lesser number of cells for desmin and nonmuscle myosin heavy chain II-B. These reactivities are similar to those observed in pulmonary LAM cells (see below)[12]. A reaction with HMB-45 and antibodies against estrogen and progesterone receptors was observed mainly in epithelioid LAM cells. These patterns of reactivity are similar to those observed in LAM cells in pulmonary lesions. However, the chylous cysts are not a feature of pulmonary LAM and are thought to result from obstruction of lymphatics.

Some of the lesions of extrapulmonary LAM have very unusual clinical and pathological manifestations. In 2 of the 22 patients studied by Matsui et al.[26],LAM affected the retroperitoneal lymph nodes and was associated with endosalpingiosis. At laparotomy. large, encapsulated masses with multiple cysts containing chylous fluid were resected from these two patients. Histologically, both were characterized by proliferating LAM cells that were histologically similar to those found in extrapulmonary LAM lesions in other patients. In both patients, some cysts were lined by ciliated epithelium resembling that of Fallopian tubes. Other cysts were lined either by flattened endothelial cells (identified by specific immunohistochemical markers) or by a mixture of these cells and epithelial cells. Many of the LAM cells, especially those subjacent to the epithelial cells, gave a positive reaction with HMB−45 antibody. Manifestations of pulmonary LAM have continued to be minimal in both patients, at 1 and 6 years after operation, respectively.

Angiomyolipomas: As mentioned above, angiomyolipomas are frequent in patients with LAM, including those in whom the disorder is associated with tuberous sclerosis. These tumors have three major components: LAM cells, which give a positive reaction with HMB−45 antibody; fragments of immature or poorly developed blood vessels, and large amounts of adipose tissue[5] (**Fig. 2 F**). The latter can impart a characteristic radiological appearance to these tumors. They can grow to very large sizes and present difficult diagnostic problems. The relationship of these tumors to other lesions of LAM is poorly understood. Similarly, their high content of adipose tissue cells, which

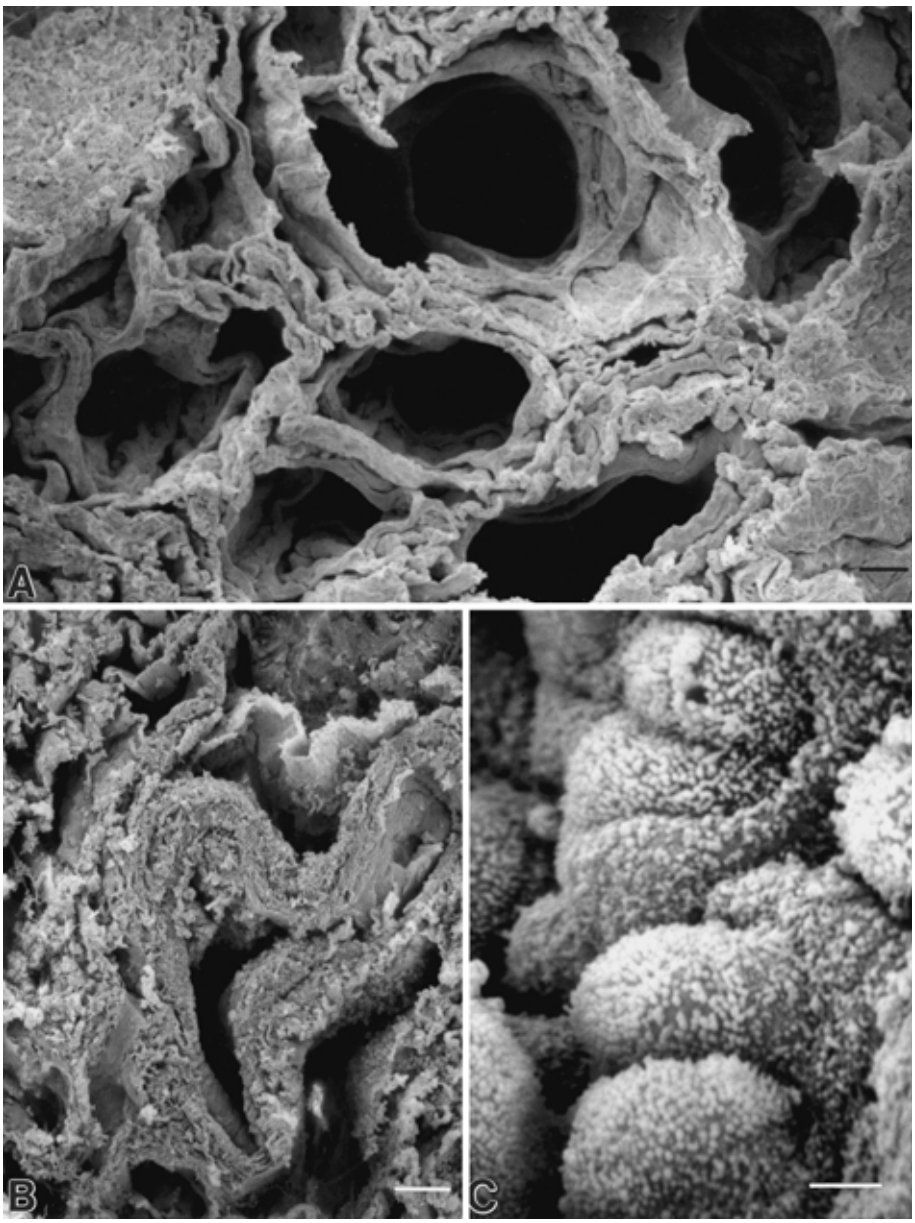


Fig. 3 Scanning electron microscopy of lung in LAM. **A**: Low magnification view of cut surface of lung, showing multiple parenchymal cysts and small nodules of LAM cells in the walls of the cysts. Compare with **Fig. 1 A**. (Bar=50 μm). **B**: A nodule of LAM cells is shown in a cross section of the wall of a cyst. (Bar=50 μm). **C**: High magnification view of luminal surface of type II pneumocyte forming the surface of a cyst. Numerous microvilli are present. (Bar=2 μm).

is not a feature of other lesions of LAM, remains unexplained.

Scanning Electron Microscopy

Scanning electron microscopic studies demonstrate the dilatation of the air spaces and the formation of cysts in lungs from patients with LAM[23] (**Figs. 3 A to 3 C**). In accord with the histologic observations cited above, the surfaces of the cysts often contained abnormally numerous type II pneumocytes. The apical surfaces of these cells are characterized by abundant microvilli (**Fig. 3 C**), thus differing clearly from those of type I pneumocytes, which are flat and lack surface projections. Nodules of LAM cells also are clearly distinguishable in cross sections of the walls of the cysts (**Fig. 3 B**). Surfaces of the pulmonary cystic lesions in LAM differ from those in pulmonary emphysema, in

which they are flat and are lined mostly by type I pneumocytes[23].

Transmission Electron Microscopy

Transmission electron microscopic studies of LAM cells are consistent with their identification as a type of smooth muscle cells. The LAM cells are surrounded by distinct basement membranes and contain variable numbers of thin (6–8 nm in diameter) cytoplasmic filaments that correspond immunohistochemically to smooth muscle F-actin[25]. In addition, they contain intermediate (10 nm) filaments, which correspond to desmin and often are distributed in the perinuclear region. Desmin filaments are limited in distribution to muscle cells[27]. However, LAM cells also give a positive reaction for vimentin, an intermediate filament protein that is known to be present in a wide variety of cells of mesenchymal origin[27]. Peripherally located dense bodies, presumed to be analogous to the Z-lines of cardiac and skeletal muscle, are present in LAM cells (**Fig. 4 A**), but are less prominent than in other types of smooth muscle cells. Such dense bodies represent insertion sites for the actin filaments. The nuclei of LAM cells usually are elongated and contain normally dispersed heterochromatin. Rough-surfaced endoplasmic reticulum often is prominent in LAM cells, as are cytoplasmic vesicles with an electron-lucent content. Mitochondria are few. The cytoplasm of epithelioid LAM cells frequently contains large numbers of glycogen particles, which can be specifically stained at the ultrastructural level (**Fig. 4 B**). These particles account for the clear appearance of large portions of the cytoplasm of these cells after staining with hematoxylin-eosin. Epithelioid LAM cells also contain cytoplasmic granules, 1–2 μm in diameter, that are surrounded by limiting membranes, and have an electron dense content characterized by a lamellar or crystalline substructure[25] (**Fig. 4 C**). On the basis of electron microscopy after immunhistochemical staining with HMB–45 antibody, these granules have been considered to correspond to stage I and II premelanosomes (see below)[28]. Premelanosome-like cytoplasmic granules also have been detected by electron microscopy in the LAM cells in angiomyolipomas[29].

Two other transmission electron microscopic observations on LAM tissues are of interest. The first concers the confirmation of the occurrence of hyperplasia of type II pneumocytes, which show typical morphological features in the lesions of LAM[23]. These features include apical microvilli, cytoplasmic bodies composed of electron-dense lamellae arranged concentrically or in parallel (lamellar bodies), and cytoplasmic projections that extend from the basal surfaces. These projections penetrate through the basement membrane, extend into the subjacent connective tissue, and make direct contacts with cytoplasmic processes of connective tissue cells. These projections are morphologically similar to those found in type II pneumocytes in normal as well as in fibrotic lungs[23].

The second observation is related to the occurrence of damage to extracellular connective tissue structures in LAM[25,30]. This damage consists of fragmentation of elastic fibers and the occurrence of collagen fibrils with a spiraling substructure. Neither of these two changes is specific for LAM, and both have been described in a variety of other pulmonary disorders.

Immunohistochemistry

Immunohistochemical studies have yielded important information on LAM cells, particularly with respect to identification by staining with HMB–45 antibody (**Fig. 5 A**), cellular proliferation and apoptosis, presence of growth factors (**Figs. 5 B and 5 D**) and receptors for steroid (sex) hormones (**Fig. 5 C**), content of muscle-specific proteins (**Fig. 5 E**), and activity of proteases (**Figs. 5 A, 5 E, and 5 F**). The observations summarized below were made on paraffin sections of formalin-fixed tissues stained by the immunoperoxidase method for single labeling and by the immunofluorescence method, followed by laser scanning confocal fluorescence microscopy, for dual labeling.

Melanosomal components of LAM cells: reactivity with HMB–45 antibody: At the present time, immunostaining with HMB–45 antibody is considered to be the most reliable method for the specific identification of LAM cells[19] (**Fig. 5 A**). However, staining with this antibody is not detectable in all LAM cells, but is localized mainly in cells of the epithelioid type. This has been a consistent finding in a large number of pa-

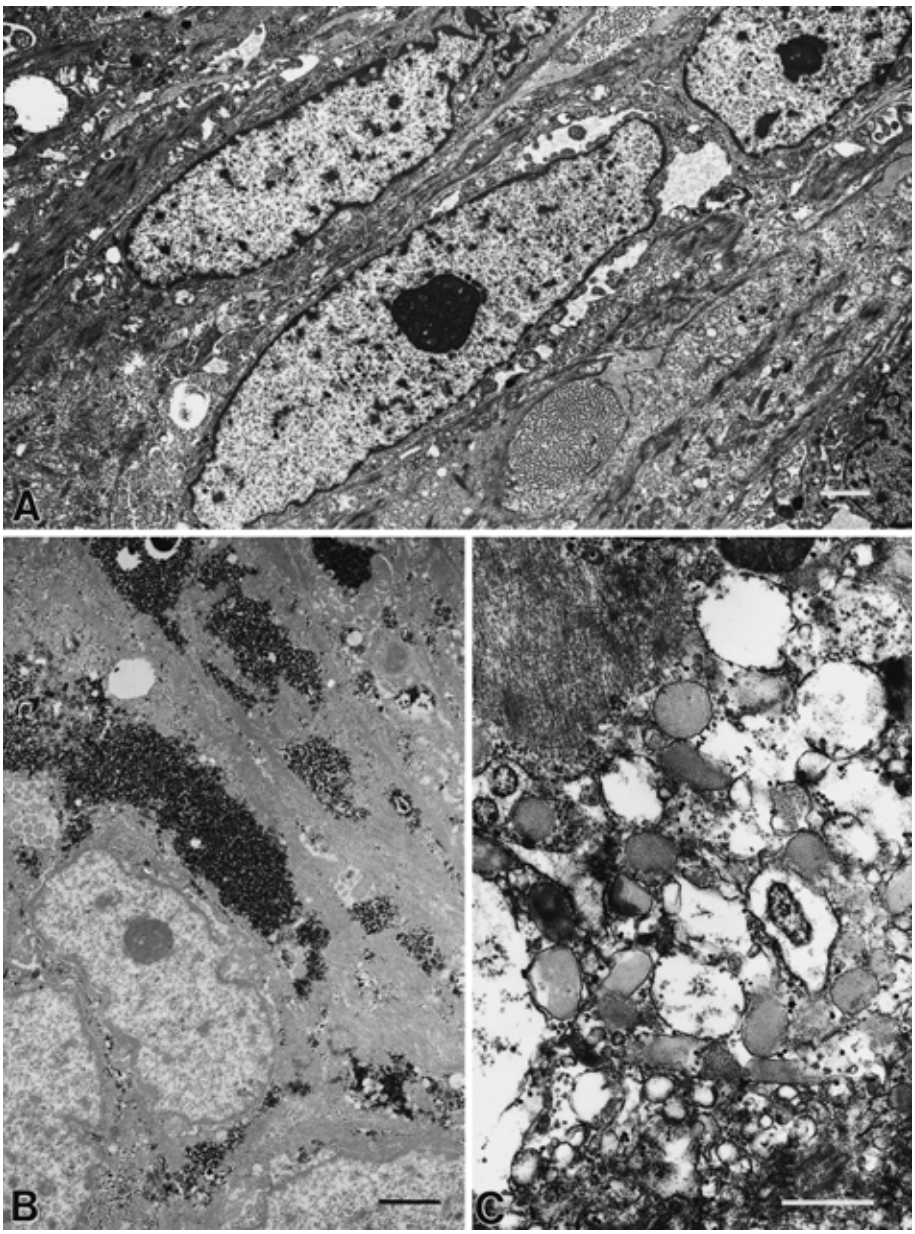


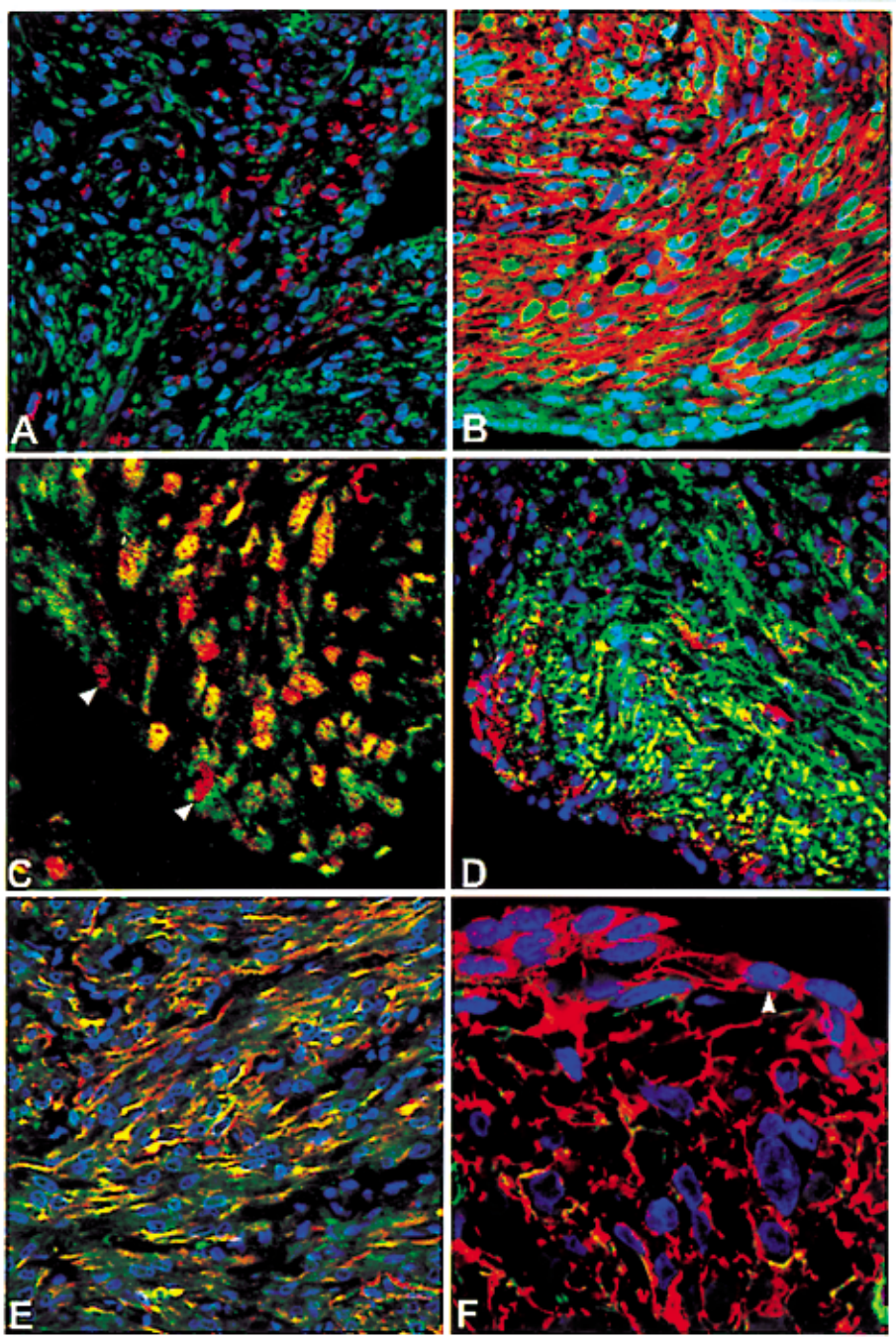
Fig. 4 Transmission electron microscopy of pulmonary LAM. **A**: Low magnification view of 3 LAM cells, showing actin-like filaments and peripherally arranged dense bodies. The cells are surrounded by bundles of collagen (Uranyl acetate and lead citrate stain, Bar=1 μm). **B**: Section stained by the Thiery method[71] for the demonstration of periodate-reactive carbohydrate materials. Numerous, highly electron dense granules of glycogen are present in the cytoplasm of an epitheliod LAM cell. (Bar=2 μm). **C**: Premelanosome-like structures consisting of parallel lamellae and crystalline array, are shown in the cytoplasm of a LAM cell (Uranyl acetate and lead citrate stain, Bar=500 nm).

tients with LAM, and the cells that give a positive reaction with HMB-45 antibody are more mature than those that are HMB-45-negative. HMB-45 is a mouse monoclonal antibody that originally was found to react with an extract from human malignant melanoma cells, and was subsequently found to also react with a variety of cells of melanocytic origin (see Matsumoto et al.[10] and Chu et al.[28] for review). The reactive sites in these cells have been identified as stage I and II melanosomes (premelanosomes) and nonmelanized portions of stage III melanosomes[28]. Gp100, the protein that reacts with HMB-45, and its alternatively spliced form (pmel 17/silver locus protein) are recognized by three different monoclonal antibodies employed

widely for the diagnosis of melanomas: HMB－45, HMB−50, and NKI/beteb. In melanocytic cells, gp100 is a part of the filamentous matrix of melanosomes[31]. This also appears to be the case in LAM cells. Immunoblots after electrophoresis of extracts from LAM tissue have demonstrated a variety of HMB−45−reactive protein bands, both smaller and larger than 100 kD. The smaller bands have been considered to represent degradation products of gp100[19].

In addition to LAM cells and melanocytic cells, only clear cell tumors have been found to be reactive with HMB−45 antibody[5]. The histogenesis of these tumors is uncertain and histochemical demonstration of large amounts of cytoplasmic glycogen is very helpful for their identification[32,33].

The evidence reviewed above and electron microscopic observations indicate, that an incomplete form of melanogenesis occurs in LAM cells. Mature melanosomes are not found in LAM cells, although they are present in other types of smooth muscle cells,



such as those in the iris[34].

Melanogenesis is the process by which dark brown cytoplasmic granules of melanin pigment are formed through the progressive oxidation and polymerization of aromatic aminoacids. The melanin pigment is produced in organelles known as melanosomes, which differentiate into four stages. Melanin itself is not present in stage I and II melanosomes (premelanosomes) but begins to accumulate in stage III melanosomes. Structural and enzymatic proteins of melanosomes are synthesized sequentially according to genetic programs and are assembled within the membrane-limited vacuoles of stage I melanosomes. Unoriented filaments and lamellae also are formed within stage I melanosomes.

Melanosomes contain several types of structural and differentiation-specific proteins, including tyrosinase, tyrosinase-related proteins (i.e., TRP-1 (gp-75) and TRP-2), gp100, MART-1/melan A[35]. Tyrosinase is a melanosomal membrane protein that is required for the synthesis of melanin. This enzyme catalyzes three distinct reactions within a single biochemical pathway: 1) the dehydroxylation of a monophenol (L-tyrosine); 2) the dehydrogenation of a catechol (L-DOPA), and 3) the dehydrogenation of a dihydroxyindol. DOPA serves as a cofactor for the first and the third of these reactions, and as a substrate for the second. The regulation of tyrosinase requires an interaction between TRPs, lysosome-associated membrane proteins, and other auxiliary enzymes[36,37]. Tyrosinase is localized in a population of coated vesicles in the transGolgi network, and in premelanosomes[38]. A positive reaction for tyrosinase was obtained only in 1 of 15 angiomyolipomas[39].

TRP-1 is of particular interest because it is the most abundant glycoprotein in human melanocytic cells[38], and is present in premelanosomes[37]. Melan A/MART-1 is a relatively small transmembrane protein that is widely distributed in melanomas, but absent from other tumors[40]. The importance of this antigen is related to its presence in angiomyolipomas[41]. However, the antibody against melan A is much less useful than HMB-45 for the diagnosis of pulmonary LAM, since only a few cases of LAM show scattered Melan A-positive cells in LAM lesions (Valencia et al., unpublished observation). Fetsch et al.[41] found a positive reaction with both MART-1 and HMB-45 antibodies in 10 angiomyolipomas. Lesions of extrapulmonary LAM from four patients were positive for HMB-45, but only one showed reactivity with anti-MART-1. Diagnostic experience with antibodies against other melanosomal proteins has been extremely limited. The functional and pathogenetic significance of melanosomal components in LAM cells has not been established. However, it is clear that melanogenesis in LAM cells is incomplete, since mature melanosomes have not

Fig. 5 Immunohistochemical reactivity of LAM cells. Confocal microscopy images showing sections stained by dual labeling procedures, using fluorescein isothiocyanate- (green) and Texas red-conjugated (red) antibodies. Colocalization of these two colors is shown in yellow. Nuclei have been counterstained with DAPI (blue), except in **Fig. 5 C**. **A**: MT-1-MMP (green) and HMB-45 (red). A cluster of HMB-45-positive cells is located in the periphery of a LAM nodule, whereas most MT-1-MMP-positive cells are seen in the central area. (×400). **B**: Melanocyte-stimulating hormone (MSH; green) and SMA (red). Staining for MSH is strong in the nuclear membranes and weak in the cytoplasm of LAM cells, where it is colocalized with the reaction for SMA (orange-yellow). (×800). **C**: Estrogen receptors (ER; green) and progesterone receptors (PR; red). Both types of receptors are preferentially localized in the nuclei of the epithelioid LAM cells, where they are frequently colocalized (yellow). Some LAM cells show staining only for PR (arrowhead) (×800). **D**: IGF-1 R (green) and HMB-45 (red). Reactivity for IGF-1 R is present in the spindle-shaped LAM cells in the central area of the nodule. HMB-45-positive cells are localized mainly in the periphery of the nodule. (×400). **E**: MT-1-MMP (green) and SMA (red). LAM cells in the center of the nodule show colocalization (yellow) of the two reactions. (×400). **F**: Type IV collagen (green) and MMP-2 (red). Reactivity for MMP-2 alone is localized in the cytoplasm of the LAM cells. Colocalization (yellow) of type IV collagen and MMP-2 is demonstrated in the basement membranes of alveolar epithelial cells and subjacent LAM cells. The epithelial basement membranes are focally disrupted (arrowhead). (×1,000).

been found in LAM cells.

Melanocortins: In an effort to understand the mechanisms that lead to the formation of premelanosomes in LAM cells, Valencia et al.[42] performed immunohistochemical studies of the distribution of melanocortins and their receptors in LAM tissue. Proteins that regulate melanogenesis, include the melanotropins (melanocyte-stimulating hormone or MSH and adrenocorticotrophic hormone or ACTH) and five types of melanocortin receptors (MCR)[43]. Valencia et al. used antibodies for the detection of human ACTH, MSH, MCR-1, MCR-2, MCR-3 and MCR-5, in lung tissue from 18 women with LAM. Normal human adrenal and pituitary glands were used as positive controls. The reactions for ACTH and MSH (**Fig. 5 B**) were moderate to strong in the nuclear membranes and moderate to weak in the cytoplasm and on the cell surfaces (**Fig. 5 E**). The cytoplasmic reactivity for MSH was stronger than that for ACTH, especially around perinuclear areas and cell surfaces of LAM cells. Among the MCR antibodies tested, only that against MCR-5 reacted with LAM cells. This reactivity was localized in the cytoplasm and cell surfaces. Dual labeling showed that the HMB-45-positive cells also were positive for either ACTH or MSH. Thus, the melanocortins are potentially involved in melanogenesis in LAM cells and may have a role in their growth and differentiation.

Factors related to cellular proliferation and apoptosis: The cause of the proliferation of LAM cells is unknown. Many factors are known to induce proliferation of smooth muscle cells, but the possible contributions of such factors to the pathogenesis of LAM have not been systematically evaluated. The numbers of LAM cells can be considered to reflect the balance between cell proliferation and cell death (for which apoptosis has to be regarded as critically important). To evaluate these relationships, Usuki et al.[44] employed immunohistochemical methods for the localization of Bcl-2 and MCL-1 (inhibitors of apoptosis), Bax (a promoter of apoptosis), c-myc (an apoptosis-related oncoprotein), proliferating cell nuclear antigen (PCNA, an indicator of mitotic activity) and nick-end labeling (to identify apoptotic cells) in lung tissues of 9 patients with LAM. In all patients, most LAM cells were Bax-positive. The LAM cells were positive for both Bcl-2 and estrogen receptors (ER) in 5 patients. Over 50% of the Bcl-2-positive LAM cells were also positive for ER. The reaction for c-Myc was positive in all patients. The immunoreactivity for Bcl-2 and MCL-1 was more intense in LAM cells than in normal vascular and bronchial smooth muscle cells. In six patients, >50% of the LAM cells were PCNA-positive. Apoptotic LAM cells were infrequent. The expression of Bcl-2 in LAM cells may be related to hormonal regulation, resulting in a very low rate of death of LAM cells by apoptosis. Additional studies by Matsui et al.[22,24] demonstrated that PCNA reactivity is high in small, spindle-shaped LAM cells, but very low in epithelioid LAM cells. Thus, it appears that LAM cells in the lung have high rates of proliferation and low rates of apoptosis. These features have not yet been evaluated in the lesions of extrapulmonary LAM or in angiomyolipomas.

Receptors for sex-steroid hormones: Several studies have demonstrated the presence of hormonal receptors in LAM tissue (see Matsui et al.[45] for review), in keeping with the concept that this disorder is under some type of hormonal regulation. Using dual labeling techniques (**Fig. 5 C**), Matsui et al. demonstrated the presence of estrogen receptors and progesterone receptors in LAM cells of the epitheliod type. Some of these cells contained only one of the two types of receptors, and approximately 50% contained both types[22]. LAM cells have some resemblances to uterine smooth muscle cells, which are known to be highly responsive to estrogen and progesterone acting through their specific receptors[22]. The relationship of hormonal receptors to the clinical features and course of LAM has been clarified only recently. Matsui et al.[22] performed immunohistochemical and confocal microscopic studies of lung tissue from 10 women with LAM to evaluate the distribution of ER and progesterone receptors (PR) in LAM cells. In 5 patients from whom tissues were obtained before hormonal treatment, PR and ER were localized mainly in the nuclei of large, epithelioid LAM cells. However, these reactions were essentially negative in similarly processed tissues from 5 patients studied after receiving therapy with progesterone and tamoxifen. It appears that PR and ER are selectively expressed in a subpopulation of LAM cells that are of the epithelioid

type, and that this expression maybe downregulated by hormone therapy. We emphasize that a negative immunohistochemical reaction for these receptors may be due to their being present in concentrations below the limit of detection by the staining method used.

Insulin-like growth factors (IGFs): The IGF system comprises IGF-1, IGF-2, their receptors (IGF-1 R and IGF-2 R) and their binding proteins (IGFBP-1 to IGFBP-6)[46]. The IGF-BPs can either increase or decrease the binding of the IGFs to their cell surface receptors. Smooth muscle cells are important targets for IGF-1 and IGF-2[47,48]. These factors are also involved in the growth and differentiation of smooth muscle cells in uterine leiomyomas and in other smooth muscle cell tumors of humans[49]. To evaluate the role of the IGF system in the proliferation of LAM cells, Valencia et al.[50] used single and dual immunohistochemical procedures for the detection of human IGF-1, IGF-2, IGF-1 R, and IGFBP-1 to IGFBP-6 in lung tissue from 20 wemen with LAM. The reactions for IGF-1, IGF-2 and IGF-1 R (**Fig. 5 D**) were positive in the cytoplasm and on cell surfaces of spindle-shaped, and some epithelioid LAM cells. The cytoplasmic reaction for IGF-1 was less intense than those for IGF-2 and IGF-1 R. Compared with LAM cells in the same tissue section, bronchial and vascular smooth muscle cells were less reactive for IGF-2 and IGF-1 R, equally reactive for IGFBP-1 and IGFBP-2, and more reactive for IGF-1. Reactivity for IGFBP-2, IGFBP-4, IGFBP-5 and IGFBP-6 was observed mainly in the cytoplasm of spindle-shaped LAM cells located in the centers of the LAM nodules. In contrast, IGFBP-5 was found mainly in the cytoplasm of epithelioid LAM cells in peripheral regions of the nodules. The reaction for IGFBP-2 was more intense than that for the other IGFBPs. These results are consistent with modulation of LAM cell proliferation through autocrine and paracrine mechanisms related to the IGF system.

Muscle proteins: In pulmonary LAM cells and normal smooth muscle cells from 14 patients, Tatsuguchi et al.[51] compared the expression of various proteins known to be present in smooth muscle. Sections were stained for α-smooth muscle actin (SMA; **Fig. 5 E**), desmin, vimentin, smooth muscle myosin heavy chains I and II, nonmuscle myosin heavy chains-A and -B, and HMB-45 antibodies. Staining for α-SMA labeled all LAM cells. However, this labeling is not specific, because SMA is present in many other cells of mesenchymal origin[52-54]. Both desmin and smooth muscle myosin heavy chains, which are markers for mature smooth muscle cells[55], also were localized in most LAM cells, but tended to be absent in the LAM cells adjacent to epithelial cells overlying the LAM nodules. Desmin is limited in distribution to muscle cells[27]. This is in contrast to vimentin, which is also present in a variety of cells other than muscle[27], and is also found in LAM cells[56]. In contrast, nonmuscle myosin heavy chain-B, which is abundant in immature or dedifferentiated smooth muscle cells (as well as in many nonmuscle cells)[57-60], was preferentially localized in subepithelial areas of LAM nodules. Nonmuscle myosin heavy chain-A was detected in only a few LAM cells, but was abundant in endothelial cells within LAM nodules. No significant relationship was observed between the distribution of HMB-45-reactive cells and those of the contractile proteins. In paraffin sections of normal lung, α-SMA, desmin, and smooth muscle myosin heavy chains were detected in all smooth muscle cells; nonmuscle myosin heavy chain A and B were present in vascular smooth muscle but not in airway smooth muscle. A few smooth muscle cells in lymphatics were positive for nonmuscle myosin heavy chain B. Under the conditions used in the study, reactivity for α-actinin could not be demonstrated in any cells. Nevertheless, peripherally located dense bodies that correspond to accumulations of α-actinin are present in LAM cells in tissue sections as well as in cultures. This study shows: 1) that LAM cells express several types of contractile proteins in addition to α-SMA, thus supporting the concept that they are smooth muscle cells and 2) that their reactivity for these proteins is heterogeneous, perhaps reflecting different degrees of differentiation of the LAM cells.

Proteases: It has become clear that the pulmonary cystic lesions in LAM are the result of proteolytic activity directed against extracellular components of the connective tissue of the lung (**Fig. 5 F**). In this respect, consideration has to be given to the possibility that the matrix metalloproteinases (MMPs) play an important role in this destruction[24]. The MMPs, their

activating enzymes (membrane-type MMPs or MT-MMPs) and their specific tissue inhibitors (TIMPs) are known to regulate the synthesis and the lysis of connective tissues in normal and pathological states[24]. Previous studies have demonstrated the importance of this system in the pathogenesis of diffuse alveloar damage, idiopathic pulmonary fibrosis, and Langerhans' cell granulomatosis, and in the formation of metastases of carcinomas of the lung[61]. To evaluate the role of MMPs and their specific TIMPs in the structural damage and cystic lesions of pulmonary LAM, Hayashi et al.[61] made immunohistochemical studies of the localization of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1, TIMP-2, HMB-45, and type IV collagens (**Fig. 5 F**) in sections of lung biopsy specimens from 4 patients with this disorder. They demonstrated greater immunoreactivity in LAM cells than in normal bronchiolar and vascular smooth muscle cells, for MMP-2 and, to a lesser extent, MMP-9 and MMP-1. MMP-2 was also localized in some elastic fibers and in the basement membranes of LAM cells and overlying epithelial cells. The basement membranes in both of these sites often showed colocalization of MMP-2 and type IV collagen. Some epithelial basement membranes showing this colocalization were disrupted. These changes were not accompanied by increased immunoreactivity for TIMPs. Taken together with previous observations showing structural damage to elastic fibers and collagen[25,62], and with the absence of demonstrable neutrophil or pancreatic types of elastase, these findings suggest that MMP-2 and MMP-9 (both of which can degrade elastin as well as collagens) are responsible for the cyst formation in LAM.

Most of the MMPs are secreted in the form of biologically inactive proenzymes, which must undergo proteolytic cleavage in order to become activated[63]. Many mechanisms can result in this activation; however, the most important of these involves proteolysis by MT-MMPs. To evaluate the role of potential activating enzymes, immunohistochemical and confocal microscopic techniques were used to localize α-SMA, HMB-45, PCNA, MMP-2, MT-1-MMP, MT-2-MMP, and MT-3-MMP in lung tissue from 10 women with LAM[24]. Tissues samples were obtained from 5 untreated patients and 5 who had been treated with progesterone and/or tamoxifen. Staining for α-SMA and MMP-2 was present in all of the LAM cells in both groups. The percentages of PCNA-, MMP-2-, or MT-1-MMP-positive LAM cells were much higher in the untreated group that in the treated group, whereas the percentages of HMB-45-reactive LAM cells were similar in the two groups. The reactions for MT-1-MMP (**Figs. 5 A and 5 E**) and PCNA were preferentially localized in small, spindle-shaped LAM cells. As in previous studies, the reaction for HMB-45 was found in large, epithelioid LAM cells. Many of the PCNA-positive cells were also positive for MT-1-MMP. Staining for MT-2-MMP and MT-3-MMP was negative. This study demonstrates that MT-1-MMP is most abundant in the LAM cells that show evidence of a high rate of proliferation and lack steroid hormone receptors. It is possible that such cells are less responsive to hormonal therapy than are the more mature epithelioid LAM cells; however, it appears that, under the conditions employed for immunohistochemical staining, this enzyme is not detected in LAM tissue from patients who have undergone prolonged hormonal thepapy. This finding can be interpreted as indicative of downregulation due to a therapeutic response.

Isolation and culture of HMB-45-Positive LAM cells

Research on LAM cells has been hampered by the lack of a tissue culture model in which biochemical and pharmacological studies can be made. Yu et al.[65] recently succeeded in isolating LAM cells (**Figs. 6 and 7**) from lung tissue of patients undergoing pulmonary transplantation for the treatment of LAM. Samples of the excised lungs were collected under sterile conditions, placed in RPMI-1640 medium at 4℃. The medium was then replaced by M-199 medium containing antibiotics and 10% fetal bovine serum (FBS). Samples of tissue were cut into 1 mm cubes, washed, and incubated at 37℃. Two to three weeks were required for growth of smooth muscle cells from the edges of the explanted tissue; these cells were HMB-45-positive, with histochemical (**Figs. 6 B to 6 D**) and ultrastructural (**Fig. 7**) characteristics similar to those of LAM cells *in situ*. As an alternative method that gave

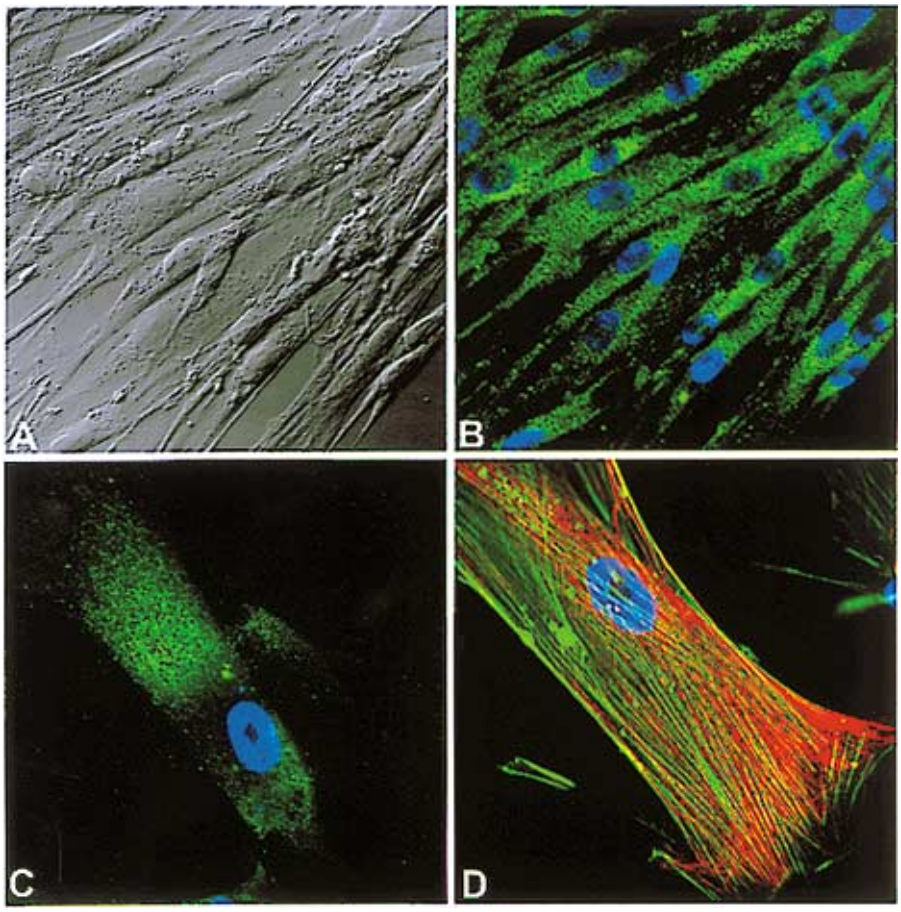


Fig. 6 Morphology and histochemistry of cultured LAM cells. The immunostained sections were counterstained with DAPI (blue). Each, ×600. **A**: Nomarski differential interference contrast micrograph of spindle-shaped LAM cells cultured from the lung of a patient with LAM. **B**: and **C**: Immunostaining with HMB-45 (green). A positive cytoplasmic reaction is evident in low (B) and high (C) passage cultured LAM cells. **D**: Dual staining of cultured LAM cells for SMA (green) and desmin (red). The staining for SMA is seen in cytoplasmic bundles and at the edges of the cells. Desmin forms a web in areas that have fewer actin bundles and in the perinuclear area.

similar results, the 1 mm cubes of tissue were trypsinized. The cells were then cultured in M-199 with 10% FBS in 100 mm dishes. Growth of LAM cells from these preparations was evident only after two weeks of culture. The reaction with HMB-45 remained positive in these cells through eight passages, but was less intense than that of the LAM cells in lung. It is expected that studies using this *in vitro* model will produce important new information on the biochemistry and pharmacology of LAM cells.

Treatment

The most widely used form of treatment of LAM utilizes progesterone[10,22]. The results of this treatment have been variable. This therapy can result only in slowing down, but not in reversal or cessation, of the disease process; however, it is associated with significant side effects [10,11]. Therapy with tamoxifen alone is uncommonly employed for LAM because the partial agonist effect of this agent on ER and the association of its use with possible exacerbation of the disease[65]. Pleurodesis can be used to minimize the risk of recurrent pneumothorax, but can cause difficult technical problems for subsequent pulmonary transplantation[10]. The latter therapy is usually performed only on patients who have very advanced stages of the disease[66]. At least three patients developed recurrent LAM in the allografted lung within two years after unilateral pulmonary transplantation[67]. In all three cases, the

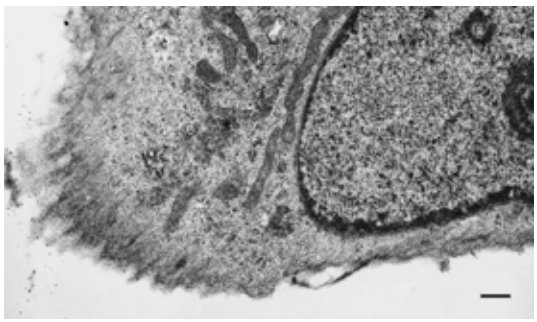


Fig. 7 Electron micrograph of a cultured LAM cell, showing well developed rough-surfced endoplasmic reticulum, actin-like cytoplasmic filaments and dense bodies (filament insertion sites) located in peripheral areas of the cell. (Uranyl acetate and lead citrate stain, Bar= 500 nm).

transplanted lung was from a male donor. In one patient, the LAM cells in the transplanted lung were studied using chromosomal markers and were found to be of donor (male) origin[68]. The frequency of occurrence of LAM in transplanted lungs needs to be evaluated in a large series of patients.

Prognosis

Early reports of LAM indicated a poor survival, with most patients dying within 10 years after the initial diagnosis (see Matsui et al.[69] for review). Recent reports suggest a better prognosis. The relationships between the histologic patterns and the survival of the patients with LAM were analyzed by Kitaichi et al.[8], who classified this disorder into two types: a predominantly cystic, and a predominantly muscular type. Their study showed that patients with the cystic type had a poorer prognosis. Using a grading system based on the overall histologic abnormalities due to cystic lesions and muscle proliferation, these investigators concluded that increased amounts of abnormal areas showed a negative correlation with survival of the patients from 2 to 5 years after open lung biopsy. The basic concept of the histologic grading system of LAM, as outlined by Matsui et al.[69], is quite similar to that proposed by Kitaichi et al.[8] in that the two major histologic features, i.e., the extent of cystic lesions and of muscle proliferation, form the basis of both grading systems. We concluded that these two major histologic findings were too variable to serve as the basis for classification into nodular and cystic types. Like Kitaichi et al.[8], Matsui et al.[69] found that the percentage of cystic lesions by itself was a predictor of survival, while the extent of infiltration by LAM cells was not. However, a combined rating of the two types of lesions provided a grading system that correlated much more closely with survival of the patients than did grading by cystic lesions alone[69].

Beasley et al.[70] studied the correlations between clinical and follow-up data and histopathologic findings in 105 women with pulmonary LAM. The actuarial survival (to pulmonary transplantation or death) of the patients from the time of lung biopsy was 85.1% and 71.0% after 5 and 10 years, respectively. The histologic severity of LAM, graded as a LAM histologic score (LHS), was determined on the basis of semiquantitative estimation of the percentage of tissue involvement by the two major features of LAM, i. e., the cystic lesions and the infiltration by LAM cells in each case: LHS-1, <25%, LHS-2, 25-50% and LHS-3, >50%. Analysis by the Kaplan-Meier method revealed significant differences in survival for patients with LHS 1, 2, and 3 ($p=0.0026$). The five and 10 year survivals were: 100% and 100% for LHS-1, 89.9% and 74.6% for LHS-2 and 59.1% and 47.3% for LHS-3. Increased degrees of accumulation of hemosiderin-laden macrophages also were associated with higher LHS scores ($p=0.029$) and a worse prognosis ($p=0.0012$). Thus, this study suggests that the LHS provides a basis for determining the prognosis of LAM.

In a very recent study, we examined over 20 histologic features in lung specimens from 174 women (mean age ± SD, 38.0 ± 8.5 years) with LAM. The LHS correlated well with many histologic variables, including the maximal size of the cysts, a random or predominantly subpleural/septal distribution of the LAM infiltrates, infiltration of the walls of blood vessels by LAM cells, degree of accumulation of hemosiderin-laden macrophages, hemorrhage, hyperplasia of type II pneumocytes and pleural fibrosis. However, the LHS did not correlate with the age of the patients at the time of diagnosis or with reactive pleuritis and formation of bullae. A predominant distribution of lesions in subpleural/septal areas was found in patients with a lower LHS. This may explain why many patients developed pneumothorax at the onset of the disease.

Thus, this study confirms the usefulness of the LHS for the evaluation of the prognosis of LAM.

In conclusion, the basic abnormalities in LAM are related to excessive proliferation of an abnormal type of smooth muscle cells (LAM cells) and to the destruction of pulmonary connective tissue by proteolytic enzymes derived from these cells. The nature (neoplastic vs. non-neoplastic) of this proliferation remains unclear. Only a sub-population of LAM cells (epithelioid cells) gives a positive reaction with HMB-45 antibody, which is the most specific marker for diagnosing the disorder. LAM cells are under some degree of control by endocrine factors, as shown by their content of steroid (sex) hormone receptors. The isolation and culture of LAM cells has provided a new method for the *in vitro* study of this disease.

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