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Targeted Approaches Toward Understanding and Treating Pulmonary Lymphangiomyomatosis (LAM)

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Abstract

Pulmonary lymphangiomyomatosis (LAM) is a rare disease found almost exclusively in women that is characterized by neoplastic growth of atypical smooth muscle-like cells in the lung, destruction of lung parenchyma, and obstruction of lymphatics. These processes lead to the formation of lung cysts, rupture of which results in spontaneous pneumothorax. Progression of LAM often results in loss of pulmonary function and death. LAM affects predominantly women of childbearing age and is exacerbated by pregnancy. The only proven treatment for LAM is lung transplantation, and even then LAM cells will often return to the transplanted lung. However, methodical and targeted approaches toward understanding LAM pathophysiology have led to the discovery of new potential therapeutic avenues. For example, the mutational inactivation of tumor suppressor complex genes *tuberous sclerosis complex 1 (TSC1)* or *TSC2* has been shown to be present in lung LAM cells. These mutations occur sporadically or in association with inherited hamartoma syndrome tuberous sclerosis (TSC). Since TSC genes function as negative regulators of the mammalian target of rapamycin (mTOR), a major controller of cell growth, metabolism and survival, rapamycin analogs have recently been used to treat LAM patients with promising results. Similarly, studies focusing on the importance of estrogen in LAM progression have suggested that anti-estrogen therapy might prove to be an alternative means of treating LAM. This mini-review summarizes recent progress in understanding LAM pathophysiology, including the latest preclinical and clinical studies, and insights regarding the role of hormones in LAM.

Keywords

interstitial lung disease; cancer; rare disease; TSC

Introduction

Lymphangiomyomatosis (LAM), a rare genetic disorder affecting primarily women of childbearing age, is an enigmatic and devastating disease with no effective treatment. The major aspect of LAM is neoplastic LAM cell proliferation in the lung, which leads to the destruction of pulmonary connective tissue and the formation of multiple cysts, rupture of which results in spontaneous pneumothorax. Interestingly, LAM occurs almost exclusively in adult women, suggesting that hormones may play an important role in the regulation of disease pathogenesis [61, 48]. LAM is associated with high incidence of angiomyoleiomas (AMLs) that occur most frequently in kidney; extrarenal sites are liver and retroperitoneum

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[78]. Pulmonary LAM can occur sporadically or in association with the inherited hamartoma syndrome tuberous sclerosis (TSC) [75, 46]. In fact, LAM is regarded as the primary form of pulmonary involvement in women with TSC. One of the major advances in understanding LAM pathology was discovering that LAM cell growth is caused at least in part by mutational inactivation of the tumor suppressor genes *Tuberous Sclerosis Complex 1* (*TSC1*) or *TSC2* [7] [73, 66, 8, 23]. The subsequent discovery that TSC1/TSC2 functions as a negative regulator of the mammalian target of rapamycin (mTOR) in LAM [27] [42] has led to the promising use of rapamycin analogs in the clinic for treatment of LAM and TSC [60] [3, 38, 10, 22]. Animal studies demonstrate a mechanistic link between TSC2 loss and cystic lung destruction in LAM [26]. The estrogen requirement for TSC2-null cell survival and metastasis [85] suggests that steroid signaling may also play a role in LAM pathogenesis, and their importance is currently the focus of intense study.

Lung pathology in LAM

In pulmonary LAM, similar to severe emphysema, the lungs contain distinctive diffuse bilateral cystic changes throughout lung parenchyma [78]. Most cysts are 0.5–2 cm in size but can be as large as 10 cm. One of the key histologic hallmarks of LAM is the presence of multinodular smooth-muscle-like LAM cells. These cells are often found at the edges of cysts or along pulmonary lymphatics, but can also be located in perivascular regions. In some instances, the pulmonary cysts are lined by LAM cells. Alternatively, LAM cell bundles at the edge of a cyst form polyp-like protrusions into the cyst lumen. In advanced disease, LAM cells infiltrate bronchioli of distal airways, vessel walls, the thoracic duct, and lymph nodes of the thorax or retroperitoneum. Fibrotic tissue is usually not abundant in LAM lesions and lung.

The origin of the LAM cell in the lung is not established [53] [9]. Unlike orderly parallel or concentric patterns normally present in smooth muscle cells of airways and vasculature, LAM cells grow in haphazard manner. Although LAM cells resemble smooth muscle cells, they are morphologically heterogeneous with shapes ranging from small, round, and spindle-like, to oval and epithelioid. LAM cell masses infiltrating into lymph nodes often grow in papillary clusters of oval and round cells. Immunohistochemically, LAM cells are similar to normal smooth muscle cells and express smooth muscle α -actin, vimentin, and desmin. LAM cells are also immunopositive for the HMB-45 (Human Melanoma Black-45) antibody that recognizes a melanocyte-related protein gp100. Melanoma associated protein CD63 and melanocytic antigen PNL2 are also detected in LAM lesions [86]. Importantly, only 20 to 70% of subjects with LAM have LAM cells that are reactive for HMB-45. Furthermore, in some cases, HMB-45-positive cells show dual reactivity for urotensin II, urotensin-related peptide, and their receptor [51]. Finally, there is an inverse correlation between reactivity for melanocytic markers and proliferating rate of LAM cells [12]. Significance of LAM cell populations with different cell lineage markers and proliferating rates remains to be established. Notably, as discussed later, estrogen and progesterone receptors have been detected primarily in the nuclei of LAM cells [62].

Deregulation of cellular signaling and its potential therapeutic targeting

The proliferative activity of LAM cells has been attributed to the loss of function of tumor suppressor complex tuberous sclerosis complex (TSC) in smooth muscle α -actin-positive LAM cells [27, 29]. TSC consists of two ubiquitously and conservatively expressed proteins called hamartin (TSC1) and tuberlin (TSC2) [42]. TSC1, a 130 kDa protein, binds ezrin-radixin-meozin (ERM) proteins, and show activity towards Rho GTPase [55]. TSC2, a 200 kDa protein, functions as a GTPase activating protein (GAP) toward the small GTPase Rheb

[43]. GAP activity of TSC2 is regulated by two types of phosphorylation, inhibitory or activatory.

Under normal growth conditions, activation of phosphatidylinositol 3-kinase (PI3K)-Akt or Erk1/2 by growth factors, cytokines and hormones results in inhibitory TSC2 phosphorylation and inactivation of TSC2 GAP activity. The phosphorylation of TSC2 disrupts interaction between TSC1 and TSC2, which then attenuates its membrane localization and its inhibition of Rheb GTPase activity [6]. This, in turn, leads to Rheb-induced activation of the mTORC1 growth-promoting signaling [6]. TSC1 or TSC2 can be also phosphorylated by PKC and IKK β leading to inhibition of TSC activity and activation of mTORC1 signaling [76, 58, 56].

In contrast, cellular stress conditions activate the GAP activity of TSC2, leading to suppression of Rheb and mTORC1 signaling. For example, under nutrient deprived conditions (low glucose and ATP levels), AMP-activated protein kinase (AMPK) and glycogen synthesis kinase 3 (GSK3) cooperatively phosphorylate TSC2 at a different site, leading to activation TSC2 GAP activity [44, 41]. GAP activation then results in Rheb GTPase inactivation and mTORC1 inhibition. Under this condition, AMPK also directly phosphorylates Raptor in mTOR complex 1 (mTORC1), and can interact with and phosphorylate kinase Ulk1 to initiate autophagy [13, 50].

In pulmonary LAM, loss of TSC1/TSC2 results in the constitutive activation of mTORC1 signaling, leading to unrestricted growth of LAM cells in the lung [27, 29]. Sensitivity of mTORC1 to rapamycin demonstrated in preclinical studies [27, 29, 30, 59] paved the way to promising use of rapamycin analogues in the clinic [60]. Rapamycin, however, induces only allosteric inhibition of mTOR without affecting mTOR catalytic activity, thus providing only partial inhibition of mTORC1. As such, although the rapamycin analog sirolimus markedly improved quality of life and stabilized pulmonary function in LAM patients, decline in pulmonary function resumed almost immediately upon cessation of treatment [3] [60]. In TSC patients sirolimus induced most AML size reduction only during the first year of treatment without a marked effect during the second year [10]. This clinical evidence underscores the limitation of rapamycin and the need for novel therapeutic target(s) for treatment of LAM [24, 52].

Loss of TSC1/TSC2 in LAM and TSC not only induces uncontrolled cell growth but also affects cell viability. Primary cultures of human cells derived from LAM lungs and TSC2-null cells demonstrated insensitivity to high doses of type I interferon β (IFN β) [31] that is known to suppress tumor progression. LAM and TSC-associated AMLs show decreased levels of type II IFN γ [16]. Requirement for IFN γ for inhibition of TSC2-null lesions was demonstrated by marked decrease of renal carcinogenesis in TSC2 $^{+/-}$ mice crossed with IFN γ transgenic mice expressing high serum levels of IFN γ [39]. Combination of rapamycin with IFN γ has greater inhibitory effects on TSC2-null cell survival as compared to a single agent [32, 17]. Upregulation of cellular signaling including STAT1 and STAT3 is also known to be involved in tumor progression. Primary cultures of human cells derived from LAM lungs and TSC2-null cells demonstrated insensitivity to high doses of IFN β [31], and increased STAT1 and STAT3 maintaining cell survival [32, 17, 19]. Reduction of STAT3 levels or activity sensitizes TSC2-null and human LAM-derived cells to growth inhibitory and pro-apoptotic affects of IFN γ [32]. Thus, IFN signaling may represent one of potential directions for an adjuvant therapy in LAM and TSC once drugs become available for clinical use.

Survival of TSC2-null cells depends on autophagy and its pharmacological inhibition prevents survival of TSC2-null cells and blocks TSC2-null tumors [63]. Thus dual inhibition

of mTORC1 signaling with rapamycin analogs and cell survival with drugs targeting autophagy may represent another potential therapeutic strategy for LAM and TSC.

Another potential therapeutic target is downstream of the multiprotein complex, mTORC2 [42]. The first established function of TORC2 was the rapamycin-insensitive cell cycle-dependent regulation of actin cytoskeleton through activation of Rho GTPase [68, 69]. Identification of mTORC2 in mammals confirmed its rapamycin-insensitive regulation of actin cytoskeleton through Rho GTPases RhoA and Rac1 [45, 65]. The Rho family of small GTPases consists of three major members including RhoA, Rac1, and Cdc42 [18]. In its GTP-bound state, activated RhoA promotes stress fiber and focal adhesion formation. TSC1 and TSC2 regulate RhoA and Rac1 GTPase activities in reciprocal fashion [34]. Overexpression of TSC1 promotes stress fiber formation through suppression of Rac1 [34] and activation of RhoA GTPase [54]. Loss of TSC2 similarly induces a rapamycin-insensitive increase in stress fiber formation due to TSC1-dependent activation of RhoA GTPase [34, 28]. Thus, in TSC2-null cells, mTORC2 modulates proliferation and survival through RhoA GTPase and Bcl2 proteins [33].

Importantly, geranylgeranylation of Rho GTPase, which ensures membrane localization and its activation [64], can be inhibited by statins, small-molecule inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [11]. Statins are a group of drugs derived either from fungi (lovastatin, simvastatin, pravastatin, and mevastatin) or made synthetically (atorvastatin and fluvastatin) with pleiotropic effects [11]. Preclinical studies of statins suggest potential differential effects on TSC2-null cells *in vitro* and *in vivo* [52]. Thus, atorvastatin inhibits growth of *TSC2*^{-/-}*p53*^{-/-} MEFs and TSC2-null ELT3 cells from Eker rat [20] but has no effect on subcutaneous tumors formed by *TSC2*^{-/-}*p53*^{-/-} MEFs *in vivo* [57]. In contrast, simvastatin by inhibiting Rho GTPase activity, promotes apoptosis of TSC2-null cells *in vitro* and in tumors *in vivo* [33]. Thus, combined therapy with simvastatin and rapamycin might be a potent means of blocking TSC2-null tumor progression. In fact, recent work using a mouse model of LAM provides exciting preclinical evidence supporting the use rapamycin and simvastatin together as a potential treatment of LAM [26]. This model recapitulates major features of human LAM including the constitutive activation of mTORC1, VEGF-D expression [81, 25], lymphangiogenesis [70], progressive destruction of lung parenchyma, and decreased survival. Most importantly, this model establishes a mechanistic link between loss of TSC2 and lung destruction in LAM. Interestingly, treatment of these mice with rapamycin inhibited TSC2-null lesion growth in the lung by a cytostatic mechanism, while simvastatin growth-inhibitory effects on TSC2-null lesions were predominantly pro-apoptotic [26]. Simvastatin markedly inhibited expression of extracellular matrix degrading enzymes metalloproteinases and prevented alveolar destruction in mouse LAM model [26]. The combination of rapamycin and simvastatin prevented both growth of TSC2-null lesions and lung destruction by inhibiting matrix metalloproteinase expression [26]. Thus, targeting both mTORC2 and mTORC1 by a combination of pro-apoptotic simvastatin and cytostatic rapamycin shows promise for combinational therapeutic intervention in LAM. However, a retrospective study of LAM patients on statins cautions about the use of statins [14]. Thus, further preclinical and clinical studies are needed to determine potential intermolecular differences of statins, their pharmacokinetics and pharmacodynamics, and safety.

Steroid Effects on LAM Progression

Several lines of clinical evidence suggest that LAM development and progression depend on estrogen signaling. First is the notable sexual dimorphism of LAM, whereby nearly all reported cases are in women. In fact, only a handful a male LAM patients have been identified, and their phenotype appears to be much less severe [21, 40, 67]. In addition,

LAM progression seems to increase during pregnancy [5], when estrogen levels are high, and possibly slows after menopause, when estrogen levels drop [47]. Similarly, LAM seems to worsen in women taking exogenous estrogens, such as oral contraception or hormone replacement [79, 71].

In addition to the aforementioned clinical evidence, *in-vitro* studies further support a role for estrogen in LAM progression. First, isolated LAM cells from the lungs of LAM patients express high levels of the estrogen receptor alpha (ER α) [62], which is known to be a potent mediator of estrogen-induced proliferation in the uterus and in breast cancer. Second, estradiol treatment of these isolated human LAM cells *in-vitro* promotes significant proliferation. Interestingly, this proliferation seems to involve both nuclear and extranuclear actions of ER α , as estradiol stimulates both transcription of proliferative genes as well as activation of Erk1/2 (most likely through transactivation of the EGF receptor [36]). In fact, MEK inhibition prevents estradiol-induced proliferation, meaning that extranuclear (nongenomic) estradiol actions are actually required for estradiol-mediated proliferation of LAM cells [82, 15]. Similarly, estradiol promotes proliferation and blocks apoptosis in cultured ELT3 leiomyoma cells [84]. These cells are derived from a uterine leiomyoma isolated from a TSC2-deficient rat called the Eker rat [80]. In fact, estrogen promotes metastasis of ELT3 cells that have been subcutaneously injected into mice [84]. As with LAM cells cultured in *in-vitro*, MEK inhibition seems to prevent the pro-metastatic effects of estradiol on ELT3 cells, again suggesting that MEK/Erk signaling, perhaps stimulated by estradiol, is required for LAM progression. Together, these data indicate that estrogen is an important promoter of LAM progression, and that anti-estrogen therapy, either through estrogen receptor blockade or aromatase inhibition, might prove to be an effective approach in treating women with LAM. In fact, a new trial called TRAIL (Trial of Aromatase Inhibition in Lymphangioleiomyomatosis) is examining the potential use of aromatase inhibitors in the treatment of LAM.

While estradiol levels are, on average, higher in women than in men, males still produce significant amounts of estrogens. In contrast, women uniquely produce another steroid that might regulate LAM: progesterone. Like ER α , progesterone receptors (PR) are expressed in human LAM cells and in ELT3 cells derived from Eker rats [62]. Unlike estradiol, it is not clear that progesterone significantly alters proliferation of LAM and leiomyoma cells *in-vitro*. In fact, clinically, progesterone is considered to be anti-proliferative in uterine leiomyoma cells, which has led to the hypothesis that progesterone might actually be useful in preventing progression of disease in LAM patients. Thus, in some occasions over the past several years, progesterone has been used as a treatment for LAM. However, a careful retrospective analysis of LAM patients treated with various progestins suggested that, in fact, progesterone had no positive, and possibly a slightly negative, effect on LAM progression [74]. Thus, exogenous progestin is currently not recommended for patients with LAM.

The origin of the LAM cell

One of the most significant mysteries in LAM concerns how LAM cells appear in the lung. Do LAM cells originate in the lung, or do they enter the lungs from other sites? Significant evidence suggests the latter: a metastatic nature of LAM [83]. The strongest of this evidence comes from LAM patients who have required lung transplantation due to progressive loss of lung function. Interestingly, many of these women develop recurrent LAM tumors in their lungs, and the new lesions are genetically identical to those that were present in the original lungs [1, 49]. These data suggest that the new lung LAM cells must have migrated to the lungs from other tumors within the body. Further proof comes from genetic analysis of lung LAM lesions. Careful analysis of TSC mutations in lung LAM cells demonstrated that they

were identical to those found in angiomyolipoma cells, but not normal kidney cells or lymphocytes, thus suggesting a metastatic nature of these TSC-null tumor cells [72, 8, 66].

If lung LAM is indeed metastatic, then the next important question centers around the origin of these LAM cells. To begin to address this question, one must start by re-addressing the sexual dimorphism of LAM. As mentioned, the sensitivity to estradiol with regard to proliferation of metastasis suggests that the higher estrogens levels in women may partially explain the almost exclusive occurrence in women. However, men still produce significant amounts of estradiol, and LAM is still discovered in anovulatory women, indicating that estradiol is not the only answer. Another possible explanation for the sexual dimorphism of LAM is related to the unique presence of a uterus in women that could serve as an origin of LAM cells. Lung LAM lesions and uterine leiomyomas share many important features, including the presence of similar appearing myometrial cell types, the expression of estrogen and progesterone receptors, and the exquisite growth sensitivity to estrogens. Interestingly, a recent study that looked carefully for the microscopic presence of LAM cells in the uterus of patients with lung LAM revealed that every patient had leiomyomas present, and all but one contained actual LAM lesions in the uterus [37].

Furthermore, LAM share similar features to another rare disease called benign metastasizing leiomyomas, a less aggressive lung disease where it is well established that uterine leiomyomas metastasize to the lungs (both lung and uterine lesions share the same genetic signatures [77, 4]), sometimes even years after uterine leiomyomas were removed surgically [35, 2]. Together, these data suggest that LAM might originate from TSC2-null myometrial cells, and that estradiol may then promote proliferation and metastasis of these myometrial cells, thus explaining the overwhelming predominance of LAM in women. Notably, as in LAM, benign metastasizing leiomyoma has been reported in a handful of men, suggesting that, in very rare situations, lung leiomyomas-like lesions can form in the lungs through other mechanisms that do not involve the uterus. Further studies are needed to better characterize the metastatic nature of LAM; however, understanding how LAM reaches the lungs will almost certainly lead to new therapeutic avenues for LAM patients.

Challenges and Future directions

As reviewed above, recent research has uncovered novel and exciting information regarding the pathogenesis and progression of LAM; however, many challenges still remain. These include definitive identification of the cell type(s) responsible for initiating LAM, and a more complete understanding regarding how steroid and mTOR signaling work together to promote LAM proliferation. More complete characterization of these important issues will undoubtedly lead to more directed and effective therapeutic interventions, and will bring hope of a cure for this rare but sometimes devastating disease.

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Figure 1.

Signaling pathways that contribute to LAM cell growth and proliferation. TSC1 and TSC2 form a complex that inhibits both Rheb and mTORC2 activity. Both estrogen and growth factors trigger receptor tyrosine kinase (RTK) activation, leading to activation of Erk1/2 and Akt. In addition to promoting ERα-mediated transcription, these two kinases also promote phosphorylation of TSC2, leading to loss of TSC1/TSC2-mediated inhibition of Rheb and mTORC2. Rheb GTPase then activates mTORC1, which promotes cell growth and proliferation via S6K1 and 4E-BP1 signaling. mTORC2 promotes RhoA GTPase activity, leading to rearrangements in the actin cytoskeleton followed by increased cell growth and survival. In contrast, low glucose levels lead to elevated AMP-activated kinase (AMPK) activity, phosphorylation of TSC2 at a different site, and increased inhibitory activity of the TSC1/TSC2 complex. In LAM cells, either TSC1 or TSC2 (usually TSC2) is inactivated, resulting in constitutive mTORC1/mTORC2 signaling and subsequent proliferation. Estradiol can also promote LAM cell proliferation through both genomic and nongenomic pathways. Notably, simvastatin, rapamycin, and inhibitors of estrogen signaling (aromatase inhibitors and ERα antagonists) can inhibit LAM proliferation at the indicated steps in the signaling pathways.