Mammalian Target of Rapamycin Signaling and Autophagy

Roles in Lymphangioleiomyomatosis Therapy

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The pace of progress in lymphangioleiomyomatosis (LAM) is remarkable. In the year 2000, *TSC2* gene mutations were found in LAM cells; in 2001 the tuberous sclerosis complex (TSC) genes were discovered to regulate cell size in *Drosophila* via the kinase TOR (target of rapamycin); and in 2008 the results were published of a clinical trial of rapamycin, a specific inhibitor of TOR, in patients with TSC and LAM with renal angiomyolipomas. This interval of just 8 years between a genetic discovery for which the relevant signaling pathway was as yet unknown, to the initiation, completion, and publication of a clinical trial, is an almost unparalleled accomplishment in modern biomedical research. This robust foundation of basic, translational, and clinical research in TOR, TSC, and LAM is now poised to optimize and validate effective therapeutic strategies for LAM. An immediate challenge is to deduce the mechanisms underlying the partial response of renal angiomyolipomas to rapamycin, and thereby guide the design of combinatorial approaches. TOR complex 1 (TORC1), which is known to be active in LAM cells, is a key inhibitor of autophagy. One hypothesis, which will be explored here, is that low levels of autophagy in *TSC2*-null LAM cells limits their survival under conditions of bioenergetic stress. A corollary of this hypothesis is that rapamycin, by inducing autophagy, promotes the survival of LAM cells, while simultaneously arresting their growth. If this hypothesis proves to be correct, then combining TORC1 inhibition with autophagy inhibition may represent an effective clinical strategy for LAM.

Keywords: tuberlin; rapamycin; chloroquine; Rheb; tuberous sclerosis

LYMPHANGIOLEIOMYOMATOSIS AND THE TUBEROUS SCLEROSIS COMPLEX GENES

Lymphangioleiomyomatosis (LAM) is a rare lung disease affecting women, with onset typically during the childbearing years (1–3). Pathologically, LAM is characterized by two distinct components: LAM cells and cystic areas of alveolar destruction. LAM cells are smooth muscle-like, express melanocytic proteins, and grow in a diffuse pattern throughout both lungs (4–6). The relationship between the LAM cells and the regions of cystic lung degeneration is unknown, although it is believed that secretion of proteases by LAM cells is a contributing factor (6–12).

LAM can occur in women with tuberous sclerosis complex (TSC), an autosomal dominant disease characterized by neuro-

logic disease (seizures, intellectual disability, autism) and benign tumors of the brain, skin, heart, and kidneys (13). About 30% of women with TSC have evidence of cystic lung disease on CT scans and are believed to have LAM, although many of these women have mild symptoms or are asymptomatic and have not had biopsy to determine whether the cystic lesions are accompanied by LAM cells (14, 15). For reasons that are not yet understood, LAM affects almost exclusively women, although a small number of men with TSC and histologically confirmed LAM have been reported (16–21).

The cloning of the *TSC1* and *TSC2* genes in 1996 and 1993, respectively, set the stage for investigations of potential links between TSC and what is now referred to as the sporadic form of LAM (LAM in women who do not meet the diagnostic criteria for TSC and who do not have germline TSC gene mutations) (22, 23). The *TSC1* gene, on chromosome 9q34, contains 23 exons, and the *TSC2* gene, on chromosome 16p13, contains 41 exons, making mutational analyses of these genes technically challenging and labor intensive, particularly in archival formalin-fixed, paraffin-embedded tissue specimens. A further challenge to genetic studies of LAM is the close admixture of LAM cells with other cell types within the lung. This makes laser capture microdissection essential in order to obtain the level of purity required for conventional DNA sequencing approaches, although next generation sequencing methods have overcome this limitation. In the relatively small number of women with sporadic LAM whose LAM cells have been isolated by laser capture microdissection and studied genetically, somatic *TSC2* gene mutations have been found in their LAM cells, but not in regions of normal lung (24). In women with sporadic LAM who also have angiomyolipomas, the same *TSC2* mutations were present in their angiomyolipomas but not in their normal kidney (24). Loss of heterozygosity of the remaining *TSC2* allele was found in both the LAM cells and the angiomyolipomas, consistent with the two-hit tumor suppressor gene model, in which inactivation of both alleles is required for tumor initiation (24, 25). *TSC2* loss of heterozygosity has also been found in LAM-like cells within lymph nodes from women who do not have pulmonary or renal manifestations of LAM. The presence of identical *TSC2* mutations in pulmonary LAM cells, lymph nodes, and angiomyolipomas from women with the sporadic form of LAM, and the presence of the same *TSC2* mutation in pulmonary LAM cells before and after lung transplantation in a woman with recurrent LAM after lung transplantation, has led to the benign metastasis model of LAM pathogenesis (26). In this model, it is hypothesized that despite their benign histologic appearance, LAM cells are capable of metastasizing to the lungs, perhaps through a multistep process promoted by estrogen-mediated cell survival of cells carrying TSC gene mutations (27), recruitment of lymphatic endothelial cells, the development of a reservoir of LAM cells within the lymphatics, and the ability of LAM cells to travel within the system circulation (28).

This discovery in the year 2000 of a genetic link between *TSC2* and sporadic LAM set the stage for two parallel lines of

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investigation, the first to understand the mechanisms through which LAM cells spread or metastasize to the lungs, which has been recently reviewed elsewhere (29), and the second to elucidate the role of mammalian target of rapamycin (mTOR) inhibition in the therapy of patients with TSC and LAM, which is the focus of this review. *TSC2* encodes tuberlin, a 220-kD protein. *TSC1* encodes hamartin, a 140-kD protein with no homology to tuberlin. Tuberlin and hamartin physically interact and function as a heterodimeric complex to inhibit the mTOR complex 1 (TORC1), which includes mTOR, Raptor, mLST8, PRAS40, and DEPTOR (Figure 1). TORC1 integrates mitogenic signals and nutrient availability with protein synthesis via substrates including p70 S6 kinase (S6K). Tuberlin inhibits TORC1 via the Ras (a small GTPase) homolog enriched in brain (Rheb), which is a key target of tuberlin's highly conserved guanine triphosphatase activating protein (GAP) domain. Tuberlin stimulates the conversion of Rheb-GTP (active) to Rheb-guanine diphosphate (inactive), thereby inhibiting TORC1.

CLINICAL RESEARCH IN LAM: A TRIUMPH OF BASIC SCIENCE DISCOVERY

The foundation for the remarkable progress in our understanding of LAM pathogenesis and the current therapeutic research for women with LAM began with a genetic screen in *Drosophila* to identify genes involved in the regulation of cell size, resulting in the first links between *TSC1/2* and TOR signaling (30–32). These links to mTOR signaling in the fly were quickly followed by experiments in mammalian cells, including evidence of mTOR activation in LAM and angiomyolipoma cells (33, 34) and in the Eker rat model of *Tsc2*, in which short-term rapamycin treatment induced apoptosis of kidney tumor cells (35).

These rapid-fire advances led to a prospective therapeutic trial in patients with LAM and TSC with angiomyolipomas, with angiomyolipoma volume as the primary endpoint. Patients received 12 months of rapamycin, followed by 12 months of follow-up after discontinuation of rapamycin (36). There was no randomization or placebo control arm in this initial study. Of 25 men and women enrolled, 20 patients completed 12 months of rapamycin and 18 patients completed the full study, including the 12 months of follow-up after rapamycin. Angiomyolipoma volume decreased to 53% of baseline after 12 months of rapamycin therapy, and after discontinuation increased to 86% of baseline at 24 months. Eighteen of the patients had LAM, 12 with TSC-associated LAM and 6 with sporadic LAM. Among these women, 24-month data were available for 10 patients, and among these, the mean FEV₁ increased by 118 ml, the FVC increased by 390 ml, and the residual volume (RV) decreased by 439 ml during the 12 months of rapamycin therapy. At 24 months, the FEV₁ was 62 ml above baseline, the FVC was 346 ml above baseline, and the RV was 330 ml below baseline, suggesting substantial sustained benefit from rapamycin.

The improved lung function in the 10 women is extremely encouraging, and perhaps unexpected: one might have expected stabilization of lung function, rather than improvement, because the degradation of lung parenchyma that accompanies LAM would not likely be correctable, at least during 12 months of therapy with a TORC1 inhibitor. Although the mechanisms of this improvement are unknown, one possibility is that shrinkage or elimination of LAM cells improves the elasticity and mechanics of the residual areas of normal lung. Importantly, however, a second study in the United Kingdom has reported interim results of a 24-month trial of rapamycin for patients with TSC and LAM (37), and found no evidence of improved FEV₁ or FVC in the four women with LAM who had completed

Figure 1. Simplified model of the tuberous sclerosis complex signaling pathway. Tuberlin and hamartin physically interact and function as a heterodimeric complex to inhibit the mammalian target of rapamycin (mTOR) complex 1 (TORC1), which includes mTOR, Raptor, mLST8, PRAS40, and DEPTOR. TORC1 integrates mitogenic signals and nutrient

availability with protein synthesis via substrates including p70 S6 kinase (S6K). Tuberlin inhibits TORC1 via the Ras homologue Rheb, which is a key target of tuberlin's highly conserved guanine triphosphatase activating protein (GAP) domain. Tuberlin stimulates the conversion of Rheb-guanosine triphosphate (active) to Rheb-guanosine diphosphate (inactive), thereby inhibiting TORC1.

12 months of rapamycin. Possible explanations for these differences include an unrecognized difference in the severity or clinical parameters of LAM between the Cincinnati and U.K. cohorts, which may be amplified by the small numbers of patients, the impact of dose reductions or cessations related to adverse events, and/or the possibility of an effort-dependent placebo effect on the results of pulmonary function testing. It is likely that clarity will be provided when the completed data are available from the U.K. trial and when results are available from the Multicenter International LAM efficacy of Sirolimus (MILES) Trial, the first prospective, randomized clinical trial in LAM, representing another landmark in the history of LAM research. This trial completed accrual in 2009.

For angiomyolipomas, the approximately 50% regression in size in the Cincinnati trial validates the importance of TORC1 signaling in the human disease. In the interim results from the U.K. trial, the volume of angiomyolipomas decreased by 13 to 42% with 12 months of rapamycin, and one patient had a 37% reduction in angiomyolipoma volume in just 2 months. The clinical implications of these results are uncertain. Because the angiomyolipomas regrew after rapamycin was discontinued in the Cincinnati trial, it appears that prolonged or intermittent therapy would be required, with the accompanying common adverse events, which include mouth ulcers and hyperlipidemia as well as the potential for infrequent but serious adverse events. Prolonged therapy is not necessarily indicated for typical angiomyolipomas, which can often be treated by arterial embolization or as a second choice, partial nephrectomy. However, if the mechanisms underlying the partial regression of angiomyolipomas were better understood, these could guide future clinical trials to develop more effective and/or shorter-term options. Most importantly, what is learned about optimal use of TORC1 inhibitors for angiomyolipomas may also guide the use of TORC1 inhibitors for LAM, because it has been demonstrated by many investigators that LAM and angiomyolipoma cells are virtually identical at the immunohistochemical and ultrastructural levels, and as we and others have now shown, carry identical *TSC2* gene mutations.

WHY DO ANGIOMYOLIPOMAS PARTIALLY REGRESS WITH RAPAMYCIN TREATMENT?

To provide a framework for considering the reasons for the partial, rather than complete, regression of angiomyolipomas, one can envision two scenarios with very different implications

Figure 2. Potential mechanisms underlying the partial regression of angiomyolipomas with rapamycin treatment. There are two possible outcomes of mammalian target of rapamycin complex 1 (TORC1) inhibition that could result in the approximately 50% decrease in angiomyolipoma size that has been observed in clinical studies: cell size could be decreased (*upper panel*) or cell number could be decreased (*lower panel*). If a decrease in cell size is accompanied by sustained TORC1 inhibition, then a further decrease in tumor size and/or elimination of angiomyolipoma cells could be achieved, hypothetically, through inhibition of autophagy or inhibition of tuberous sclerosis complex-2-dependent, rapamycin-independent pathways. If a decrease in cell number is accompanied by evidence of TORC1 activation, then a further decrease in tumor size and/or elimination of angiomyolipoma cells could be achieved by targeting TORC1 via other agents. In human lymphangioleiomyomatosis, these two scenarios may coexist, based on regional differences in bioenergetic stress and hypoxia.

biologically and clinically: either the tumors regress by 50% because cells have decreased in size as a consequence of sustained TORC1 inhibition, or the tumors have regressed by 50% because there are fewer cells, some of which may have escaped TORC1 inhibition (Figure 2). There are two possible outcomes of TORC1 inhibition that could result in the approximately 50% decrease in angiomyolipoma size that has been observed in clinical studies: cell size could be decreased (Figure 2, *upper panel*) or cell number could be decreased (Figure 2, *lower panel*). If a decrease in cell size is accompanied by sustained TORC1 inhibition, then a further decrease in tumor size and/or elimination of angiomyolipoma cells could be achieved, hypothetically, through inhibition of autophagy or inhibition of TSC2-dependent, rapamycin-independent pathways. If a decrease in cell number is accompanied by evidence of TORC1 activation, then a further decrease in tumor size and/or elimination of angiomyolipoma cells could be achieved by targeting TORC1 via other agents. In human LAM, these two scenarios may coexist, based on regional differences in bioenergetic stress and hypoxia. We will consider them separately for the purpose of understanding how best to use TORC1 inhibitors for patients with LAM and TSC.

Scenario 1:

Sustained TORC1 inhibition and overall decrease in cell size results in a 50% decrease in angiomyolipoma volume. This would suggest that rapamycin has had the expected cytostatic response and decrease in cell size, but the tumor cells have

survived the 12 months of rapamycin therapy, facilitating regrowth after rapamycin is discontinued. The mechanistic considerations of this scenario include (1) Rapamycin-independent functions of TORC1. It is increasingly clear that rapamycin does not inhibit all functions of TORC1 (38–40), and the development of TOR kinase inhibitors may provide new therapeutic options in TSC and LAM. (2) TORC1-independent functions of TSC2. Currently, Rheb is the only generally accepted target of the TSC1/TSC2 complex, and TORC1 is the only generally accepted target of Rheb. However, we and others have observed functions of TSC2 and Rheb that are clearly rapamycin-insensitive (38–44) and may prove to be TORC1-independent. Karbowiczek and colleagues found that Rheb's inhibition of B-Raf activity (41) and B-Raf and C-Raf heterodimerization (43) are resistant to rapamycin treatment. Lee and colleagues found that Tsc1/2-null cells exhibit increased MMP-2 expression and activity, which are insensitive to rapamycin treatment (44). Targeting these pathways in combination with TORC1 could have synergistic benefit in angiomyolipoma and LAM cells. (3) TORC1-dependent pathways that are induced by rapamycin and promote the survival of the TSC2-null cells. Interestingly, rapamycin treatment would be predicted to reactivate several

Figure 3. Effects of dysregulation of autophagy in tumorigenesis and in lymphangioleiomyomatosis (LAM). LAM cells are predicted to have low levels of autophagy, because of mammalian target of rapamycin complex 1 activation. This may have a dual effect in tuberous sclerosis complex and

LAM, by enhancing tumorigenesis via increased production of reactive oxygen species, and inhibiting tumorigenesis via decreased survival under conditions of bioenergetic stress, including hypoxia.

Figure 4. Potential therapeutic strategy combining mammalian target of rapamycin complex 1 (TORC1) inhibition and autophagy inhibition in lymphangioleiomyomatosis (LAM). We hypothesize that rapamycin blocks the further growth of LAM cells by inhibiting protein translation, but simultaneously promotes the survival of LAM cells by inducing autophagy. If this hypothesis is correct, then combined inhibition of TORC1 and an autophagy inhibitor such as chloroquine could have synergistic efficacy. Other therapeutic combinations also need to be considered, as outlined in Figure 2.

signaling pathways that are down-regulated in TSC2-null tumor cells, including autophagy. Reactivation of these pathways may contribute to the survival of the cells during rapamycin treatment, and targeting these pathways in combination with rapamycin could lead to a highly effective treatment for angiomyolipoma and LAM.

Scenario 2:

Loss of TORC1 inhibition with overall decrease in cell number, resulting in a 50% decrease in tumor volume. Mechanistic considerations underlying this situation would include (1) unequal drug delivery, which may be complex in an angiomyolipoma in which vascular aneurysms and abnormalities are typical and in which vascular flow is disrupted; or (2) the development of a subpopulation of rapamycin-resistant cells. A disconnect between phospho-ribosomal protein S6 and phospho-4EBP1 may point toward a recently identified mechanism through which sustained use of rapamycin *in vitro* leads to unequal inhibition of the downstream targets of TORC1 (38–40).

AUTOPHAGY IS REGULATED BY TORC1, AND CAN BOTH PROMOTE AND INHIBIT TUMORIGENESIS

Autophagy (from the Greek, “auto” oneself, “phagy” to eat) is a cellular degradative pathway that involves the delivery of cytoplasmic cargo to the lysosome. At least three forms have been identified—chaperone-mediated autophagy, microautophagy, and macroautophagy—that differ with respect to their physiological functions and the mode of cargo delivery to the lysosome (45). Autophagy occurs at low basal levels in all cells to perform homeostatic functions (46) and is rapidly up-regulated in response to metabolic stresses, resulting in the recycling of organelles and other cytoplasmic substances to provide metabolic precursors (47). Autophagy controls mitochondria turnover by removing dysfunctional mitochondria and protects cells from reactive oxygen species (ROS)-induced damage and from the release of proapoptotic mitochondrial proteins (48).

TORC1 is a key inhibitor of autophagy, regulated by multiple upstream factors including oxygen, amino acids, glucose, and growth factors (49–55). In yeast, multiple genes essential for autophagy (referred to as ATG genes) act downstream of TOR (56, 57). ATG proteins are conserved in mammals and function to induce the generation, maturation, and recycling of autophagosomes (58). The precise mechanisms through which mTOR regulates autophagy remain an area of active investigation. In recent work, mTOR was found to interact with a complex with three essential autophagy genes, Atg13-FIP200-ULK, and to directly phosphorylate and regulate Atg13 and ULK, thereby providing a direct link between mTOR activity and autophagy in mammalian cells (55, 59, 60).

Finally, it is important to emphasize that TORC1 is not the only regulator of autophagy in mammalian cells (45). For example, P53 positively regulates autophagy through up-regulation of DRAM (damage-regulated autophagy modulator), a lysosomal protein that may induce autophagy (61), and Bcl-2 and Bcl-XL inhibit autophagy by binding to Beclin 1 autophagy protein (62).

Autophagy appears to play a complex and context-dependent role in tumor development (63). One of the first specific links between the autophagy machinery and tumorigenesis arose from mice with heterozygous disruption of Beclin 1 (ATG6), which is part of a complex required for autophagic vesicle formation. These mice have decreased autophagy, develop spontaneous lymphomas, lung carcinomas, and hepatocellular carcinomas, and undergo accelerated hepatitis B virus-induced carcinogenesis (64, 65). Interestingly, Beclin 1 is monoallelically

deleted in a high percentage of human breast, ovarian, and prostate cancers, and decreased expression of Beclin 1 has been reported in human breast, ovarian, and brain tumors (66). Knockout of Bif-1, which forms a complex with Beclin-1, also enhances the development of spontaneous lymphomas and solid tumors in mice (67). These genetic data indicate that lower levels of autophagy can promote tumorigenesis. This may be related to the fact that autophagy promotes the removal of damaged mitochondria thereby lowering levels of ROS (68). Autophagy-defective tumor cells have higher levels of ROS and genome damage with stress (69, 70) and accumulate p62. Importantly, p62, which has been used as a marker of autophagy inhibition, was recently discovered to have an independent role in promoting tumorigenesis in cells with defective autophagy through regulation of NF- κ B (71). The contribution of p62 accumulation to tumorigenesis in diseases associated with the loss of direct mTOR regulators, such as TSC and LAM, is not known. Finally, autophagy may play a direct role in cell growth, because Beclin 1 expression slows the proliferation of tumor cell lines, associated with a decrease in cyclin E and phosphorylated Rb, without affecting cell death (72).

Although these genetic data suggest that defective autophagy promotes tumorigenesis, inhibiting autophagy paradoxically blocks the growth of established tumors in certain animal models, likely by promoting survival in situations of bioenergetic stress (45, 52, 73). Chloroquine or hydroxychloroquine, which are often used to inhibit autophagy in animal studies, are 4-aminoquinoline drugs that passively diffuse into lysosomes and block fusion of the autophagosomes with the lysosomes. In a model of Myc-induced lymphoma, chloroquine treatment blocked tumor progression by twofold (49). Although a great deal of additional preclinical investigation is needed, autophagy-targeted agents have a clear potential to contribute to the suppression of human tumors.

WILL AUTOPHAGY-TARGETED THERAPIES BE EFFECTIVE IN LAM?

Because it is well-established that LAM cells and TSC tumor cells have hyperactivation of TORC1, it is likely that autophagy is dysregulated, although this has not yet been directly studied. Recently Zhou and colleagues found that Tsc1^{-/-} mouse embryonic fibroblasts (MEFs), with high mTORC1 activity, had lower levels the autophagy marker LC3-II than Tsc1^{+/+} MEFs (74). Finlay and coworkers have found that Tsc2-null cells have enhanced levels of ROS (75), which could be due to delayed clearing of mitochondria because of autophagy inhibition. The development of autophagy markers that could be used in tumor sections would allow several key questions to be addressed. (1) Is autophagy low in human LAM and angiomyolipomas and/or in tumors in animal models of TSC, and is it uniformly low, or do hypoxic regions or other areas with bioenergetic stress have up-regulation of autophagy (Figure 3)? It is known that autophagy localizes to hypoxic tumor regions where it is believed to support cell survival (69, 76). (2) Do TORC1 inhibitors induce autophagy, and again is this uniform or regional? (3) Does sustained treatment with TORC1 inhibitors lead to sustained autophagy induction? The feedback signaling loops through which TORC1 inhibition can lead to reactivation of PI3K and Akt signaling (77–80) and the existence of rapamycin-insensitive functions of TORC1 (38–44) make it critical to address these questions *in vivo*. (4) Does combinatorial treatment with TORC1 inhibitors and autophagy inhibitors result in enhanced cell death, and is this effect more pronounced within regions of bioenergetic stress (Figure 4)? Ultimately, the goal is to implement effective therapeutic

strategies to preserve lung function in LAM, for which therapies are urgently needed.

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