CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Cathepsin K in Lymphangioleiomyomatosis

LAM Cell–Fibroblast Interactions Enhance Protease Activity by Extracellular Acidification

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Lymphangioleiomyomatosis (LAM) is a rare disease in which LAM cells and fibroblasts form lung nodules and it is hypothesized that LAM nodule–derived proteases cause cyst formation and tissue damage. On protease gene expression profiling in whole lung tissue, cathepsin K gene expression was 40-fold overexpressed in LAM compared with control lung tissue ($P \leq 0.0001$). Immunohistochemistry confirmed cathepsin K protein was expressed in LAM but not control lungs. Cathepsin K gene expression and protein and protease activity were detected in LAM-associated fibroblasts but not the LAM cell line 621-101. In lung nodules, cathepsin K immunoreactivity predominantly co-localized with LAM-associated fibroblasts. *In vitro*, fibroblast extracellular cathepsin K activity was minimal at pH 7.5 but significantly enhanced at pH 7 and 6. 621-101 cells reduced extracellular pH with acidification dependent on 621-101 mechanistic target of rapamycin activity and net hydrogen ion exporters, particularly sodium bicarbonate co-transporters and carbonic anhydrases, which were also expressed in LAM lung tissue. In LAM cell–fibroblast co-cultures, acidification paralleled cathepsin K activity, and both were reduced by sodium bicarbonate co-transporter ($P \leq 0.0001$) and carbonic anhydrase inhibitors ($P = 0.0021$). Our findings suggest that cathepsin K activity is dependent on LAM cell–fibroblast interactions, and inhibitors of extracellular acidification may be potential therapies for LAM. (*Am J Pathol* 2017, 187: 1750–1762; <http://dx.doi.org/10.1016/j.ajpath.2017.04.014>)

Lymphangioleiomyomatosis (LAM) is a lung and lymphatic disease that may lead to respiratory failure. In LAM, the lung parenchyma is progressively replaced by cysts surrounded by heterogeneous groups of cells.¹ These groups of cells, termed *LAM nodules*, contain LAM cells, which are clonal and have inactivating mutations in either *TSC1* or, more often, *TSC2*. The protein products of *TSC1* and -2, hamartin and tuberlin, respectively, form a heterodimer that, by inactivating the small GTPase Rheb, in turn suppresses the activity of the mechanistic target of rapamycin (mTOR).² In LAM cells, constitutive activation of mTOR leads to abnormal proliferation, migration, inhibition of autophagy, and metabolic dependence on glycolysis.^{3–6} LAM cells express markers of both smooth muscle, including α -smooth muscle actin, and melanocyte lineages with microphthalmia transcription factor, glycoprotein 100,

and PNL2.⁷ This mixed phenotype is characteristic of the perivascular epithelioid cell group of neoplasms.⁸ Genetic, and more recently, histologic studies have shown that LAM nodules also contain a significant population of recruited wild-type cells⁹ including fibroblasts,¹⁰ mast cells,¹¹ and other inflammatory cells.¹²

The mechanism of cyst formation is not well understood, although lung cysts are thought to arise as a result of LAM nodule–derived matrix degrading proteases.² The expression of various protease families has been described

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in LAM. The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases with roles in many biological processes, including extracellular matrix turnover, inflammation, angiogenesis, metastasis, regulation of growth factor, and chemokine activity.¹³ LAM lung nodules express MMPs 1, 2, and 14.^{14–16} MMP-2 is overexpressed by *TSC2* knockout cells,¹⁷ and we and others have shown that women with LAM have higher levels of MMP-2 and -9 in serum and MMP-9 in urine than do healthy women.^{18–20} However, in a recent study, MMP inhibition with doxycycline did not reduce the decline in lung function despite the suppression of MMP-9, suggesting that other proteases are involved in lung destruction.²⁰ The serine protease plasmin is increased in LAM lung, whereas its inhibitor, plasminogen activator inhibitor 1, is reduced, suggesting activation of this protease axis.²¹ Cathepsin K is a cysteine protease that is expressed in LAM lung nodules and other perivascular epithelioid cell neoplasms.^{22,23} Unlike the MMPs and plasmin, cathepsin K is not present in normal lung tissue, but is classically expressed by osteoclasts as a bone-remodeling protease²⁴ and by tumor stromal fibroblasts.²⁵ Cathepsin K requires low pH for its activation. Inside the cell, this generally occurs in lysosomes, whereas in tumor stroma, cathepsin K activation is dependent on acidification of the extracellular space by membrane transporters including carbonic anhydrases (CAs), vacuolar-type H⁺-ATPases, and sodium bicarbonate co-transporters.^{26–28}

Here we investigated the expression of cathepsin K and the mechanism of cathepsin K activation by extracellular acidification using *in vitro* models of LAM and LAM lung tissue.

Materials and Methods

Patients and Tissue

Women with LAM receiving clinical care at the National Centre for LAM (Nottingham, UK) were enrolled in a comprehensive cohort study. Informed consent was obtained for the use of tissue samples collected as a part of clinical care, including diagnostic biopsy or diseased LAM lung removed at the time of lung transplantation to be used for cell and tissue culture. LAM lung tissue removed at the time of transplantation was received from UK transplant centers and the National Disease Research Interchange (Philadelphia, PA). The study protocol was approved by the Nottingham research ethics committee (ref. number 13/EM/0264), and written informed consent was obtained from all patients.

Cell Isolation and Culture

Fibroblast-like cells, now termed *LAM-associated fibroblasts* (LAFs), were obtained from collagenase-digested fresh LAM lung tissue, cultured in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DME-F12; Life Technologies Ltd, Paisley, UK) and were used between

passages 3 and 6. LAFs do not have *TSC* mutations, and they express full-length tuberlin protein and suppressible mTOR activity in the absence of serum, consistent with wild-type cells, as previously described.¹⁰ 621-101 Cells were derived from the renal angiomyolipoma of a patient with sporadic LAM, have inactivation of both alleles of *TSC2*, express estrogen receptors α and β ,²⁹ and were a gift from Elizabeth P. Henske (Brigham and Women's Hospital, Harvard Medical School, Boston, MA). These cells were maintained in DME-F12 with 10% fetal calf serum. *TSC2*^{-/-} and *TSC2*^{+/-} murine embryonic fibroblasts (MEFs) were a gift from David Kwaitkowski (Brigham and Women's Hospital, Harvard Medical School, Boston, MA) and were derived as described by Onda et al.³⁰ Normal human lung fibroblasts from premenopausal female donors were purchased from Lonza (Slough, UK) and Promocell (Heidelberg, Germany) and were maintained in DME-F12 with 10% fetal calf serum.

Cell and Tissue Models

Co-cultures were established either in 12-well Boyden chamber transwells or as direct-contact co-cultures. In the transwell system, LAFs and 621-101 cells were incorporated in a 10:1 ratio. Polycarbonate membrane transwell inserts (0.4- μ m pore size; Corning Life Sciences, SLS, Nottingham, UK) were equilibrated for 1 hour at 37°C and 5% CO₂ before cells were added. LAFs were seeded at 5×10^5 cells/mL in the lower chamber and 621-101 cells at 5×10^4 cells per 500 μ L in the upper chamber. Monocultures of both cell types maintained the same cell number as did co-cultures. Direct-contact LAF and 621-101 co-cultures were set up using a total of 5×10^4 cells in a 1:1 ratio. A mixture of cells was resuspended in serum-free DME-F12 and then cultured in tissue culture-treated plastic. Monocultures of both cell types were set up using 5×10^4 cells/well. For pH measurement, 5×10^4 621-101 cells, *TSC2*^{-/-} MEFs (rapamycin- or vehicle-treated), or *TSC2*^{+/-} MEFs were cultured in 24-well tissue culture plates.

Fresh *ex vivo* LAM lung tissue obtained from transplant lungs was washed thoroughly in Dulbecco's phosphate-buffered saline (PBS) (Sigma, Dorset, UK) and Dulbecco's modified Eagle's medium containing penicillin/streptomycin/amphotericin B (Sigma). Tissue from different areas in the lung parenchyma was cut into 3-mm cubes and placed in 24-well tissue culture plates. Tissue was equilibrated overnight in serum-free DME-F12, after which it was treated with vehicle or 10 nmol/L rapamycin or 10 nmol/L estrogen or both in serum-free DME-F12 for 48 hours.

MTT Assay

A MTT 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay reduction was performed to assess cell viability after treatment with low-pH media or inhibitors of mTOR or membrane transporters and proton

Figure 1 Relative protease gene expression in human lymphangioleiomyomatosis (LAM) and control lungs. **A:** Whole-lung RNA was extracted from six patients with LAM and three control patients without LAM. Protease gene expression was analyzed by real-time quantitative PCR. Protease gene expression is normalized to β -actin. **B:** Lung tissue was incubated for 48 hours with 10 nmol/L rapamycin, 10 nmol/L estrogen, estrogen and rapamycin, or vehicle, and gene expression was measured by quantitative RT-PCR. Data are expressed as means $\pm$ SD. $n = 6$ independent parenchymatous areas each of 2 LAM donors assessed for gene expression after treatment. * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$.

pumps. LAFs, 621-101 cells, or $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs cultured in unbuffered medium were treated with a sterile 0.5 mg/mL MTT solution (Sigma) for 4 hours at 37°C and 5% CO₂. Remaining MTT solution was discarded after 4 hours, and the resulting formazan crystals were dissolved in propan-2-ol. Samples were then transferred to a 96-well plate, and absorbance was read at 570 nm with a background subtraction of 690 nm.

Quantification of Gene Expression

Total RNA was extracted from 10⁶ LAFs, normal human lung fibroblasts, or 621-101 cells cultured in 6-well tissue culture plates for 24 hours using the GenElute Mammalian Total RNA Miniprep Kit (Sigma). RNA from treated and untreated tissue explants was extracted by first homogenizing the tissue using an IKA T 25 Ultra-turrax Homogenizer (IKA, Oxford, UK), followed by shearing, centrifuging, and filtering to remove tissue debris. Contaminating genomic DNA was removed using the

On-Column DNase I Digestion Set (Sigma). cDNA was synthesized using the Superscript III First-Strand Synthesis System (InvitroGen, Life Technologies) with random hexamer primers as per the manufacturer's instructions. Levels of relative gene expression of *MMP1*, -2, -9, -12, -13, and -14; tissue inhibitor of metalloproteinases 1 to 3; cathepsins B, D, H, K, L, and S; urokinase-type plasminogen activator; urokinase-type plasminogen activator receptor; plasminogen activator inhibitor 1; ADAM metalloproteinase domain 17; and calpains 1 and 2 were determined by amplifying cDNA via real-time quantitative PCR using the Brilliant III SYBR Green qPCR Master Mix (Agilent Technologies, Cheshire, UK). Predesigned and validated KiCq Start SYBR Green Primers (Sigma) were used. Primers were selected on the basis of their rank and exon locations. Reactions were performed in triplicate. Expression levels of target genes were determined relative to a housekeeping gene β -actin using the comparative C_T ($2^{-\Delta C_T}$) method.³¹

IHC Analysis and Immunofluorescence

Immunohistochemistry (IHC) analysis was performed on formalin-fixed, paraffin-embedded sections. After deparaffinization, antigen retrieval, where required, was performed in sodium citrate buffer solution, pH 6.0, for 20 minutes in a steamer. Sections were then blocked with 3% hydrogen peroxide (Sigma) followed by 2.5% horse

Figure 2 Expression of cathepsin K in lymphangioleiomyomatosis (LAM). IHC analysis of three representative LAM lung tissues. α -Smooth muscle actin (SMA) identifies LAM nodules (arrowheads) adjacent to cysts, which have staining positive for cathepsin K (CTSK). Original magnification, $\times 2.5$. Scale bars = 1 mm.

Figure 3 Cathepsin K (CTSK) expression within lymphangioleiomyomatosis (LAM) lung nodules. IHC staining for CTSK, α -smooth muscle actin (SMA), fibroblast surface protein (FSP), and melanoma marker antibody (PNL2) in samples from two patients with LAM and normal lung tissue. Original magnification, $\times 10$. Scale bars = 200 μ m.

serum (Vector Laboratories, Peterborough, UK) before incubation with primary antibody at 4°C overnight. Sections were washed in PBS with 0.05% Tween 20 then incubated with secondary antibody for 1 hour at room temperature. Chromogenic detection was performed using ImmPact DAB (Vector Laboratories). For double chromogenic IHC analysis, following ImmPact DAB incubation, sections were blocked, incubated with the second primary antibody, washed in PBS with 0.05% Tween 20, and then incubated with secondary antibody for 1 hour at room temperature. Chromogenic detection for second antibody was performed using Vector Blue Alkaline Phosphatase Substrate (Vector Laboratories). Levamisole was added to block endogenous alkaline phosphatases. Sections were counterstained with Mayer's hematoxylin (Sigma) and mounted using Vecta-Mount (Vector Laboratories). For double-fluorescence IHC analysis, formalin-fixed, paraffin-embedded sections were sequentially incubated with primary antibodies against both

antigens, followed by washing and then incubation with both fluorophore-conjugated secondary antibodies (pre-adsorbed against the other species), counterstaining with DAPI, and mounting in fluorescent mounting medium (Dako UK Ltd, Ely, UK).

Immunofluorescence detection of proteins was performed in cultured cells grown on the 8-well Nunc Lab-Tek II Chamber Slide System (Fisher Scientific, Loughborough, UK) in DME-F12 with 10% fetal calf serum for 24 hours. The cells were then fixed in 4% formaldehyde overnight at 4°C, washed in PBS, then permeabilized in 0.15% Triton $\times 100$ in PBS for 10 minutes at room temperature. Samples were blocked in 10% goat serum and incubated with primary antibodies overnight at 4°C, followed by incubation with fluorophore-conjugated secondary antibodies for 1 hour at room temperature in the dark. Samples were incubated with DAPI and mounted in fluorescent mounting medium (Dako).

Figure 4 Expression and activity of cathepsin K in cultured cells. **A:** Real-time quantitative PCR analysis of cathepsin K in lymphangioleiomyomatosis-associated fibroblasts (LAFs), normal human lung fibroblasts (NHLFs), and 621-101 cells. **B:** Immunofluorescence detection of cathepsin K protein in two LAFs, NHLFs, and 621-101 cell cultures. **C:** Intracellular cathepsin K activity is visible as red fluorescence in two LAF cultures. Fluorescence is reduced by the cathepsin K-specific inhibitor (CTSK inh) L006235 and completely abrogated by the cysteine protease inhibitor E64. Data are expressed as means $\pm$ SD (**A**). $n = 3$ (**A**, NHLFs), $n = 4$ (**A**, LAFs). $*P < 0.05$. Original magnification, $\times 20$ (**B** and **C**). Scale bars = 200 μ m (**B** and **C**).

Primary antibodies used were mouse anti-cathepsin K (3F9 ab37259; Abcam, Cambridge, UK), 1:2000 (IHC) and 1:100 (immunofluorescence); rabbit anti-cathepsin K (11239-1-AP; Proteintech, Manchester, UK), 1:500 (IHC-F)

and 1:1000 (IHC); anti- α -smooth muscle actin (1A4, A2547; Sigma), 1:10,000; anti-fibroblast surface protein (1B10, F4771; Sigma), 1:50; anti-melanoma-associated antigen PNL2 (MSK082; Zytomed, Berlin, Germany), 1:50; rabbit anti-CA IX (ab15086; Abcam), 1:500; and rabbit anti-solute carrier family 4 member 4 (SCL4A4, ab187511; Abcam), 1:2000. Secondary antibodies used were Vector ImmPress horseradish peroxidase anti-mouse and anti-rabbit antibody (Vector Laboratories), Alexa Fluor 488 goat anti-mouse IgG antibody, Alexa Fluor goat anti-rabbit IgG antibody (Fisher Scientific), anti-Mouse IgM peroxidase conjugate (Sigma).

Cathepsin K Activity Assays

Intracellular cathepsin K activity was recorded in live cells using the Magic Red substrate (ImmunoChemistry Technologies, 2B Scientific, Bicester, UK). Cultured cells were grown on the 8-well Nunc Lab-Tek II Chamber Slide System in DME-F12 serum free for 24 hours. Cells were then treated with unbuffered Dulbecco's modified Eagle's medium, pH 6.5, for 2 hours, after which Magic Red substrate was added to the media at a 1:26 ratio in the presence and absence of 100 nmol/L cathepsin K inhibitor L006235 and 10 μ mol/L cysteine protease inhibitor E64 (Tocris, Abingdon, UK). The cells were then incubated for 16 hours at 37°C and 5% CO₂. Cells were washed in PBS and nuclei labelled using 0.5% v/v Hoechst stain for 10 minutes at 37°C and 5% CO₂. Samples were then mounted using PBS.

Extracellular cathepsin K activity was measured using a Cathepsin K Activity Fluorometric Assay Kit (Abcam). Indirect contact co-cultures and monocultures were run as described in *Cell and Tissue Models*. Cells were cultured in 12-well plates and transwell inserts in unbuffered Dulbecco's modified Eagle's medium supplemented with 0.584 g/L L-glutamine and 0.004 g/L folic acid at pH 6.0, 7.0, and 7.5 for 48 or 96 hours. Media were then harvested, clarified, and concentrated fivefold using Vivaspin 2 Centrifugal Concentrators (Sartorius, SLS, Nottingham, UK). Ac-LR-AFC substrate (200 μ mol/L) was added to each concentrated media sample with cathepsin K reaction buffer. Samples were incubated in a black-walled 96-well plate at 37°C for 16 to 18 hours in the dark, and fluorescence was read at a 400-nm excitation and 505-nm emission.

pH Measurement

Unbuffered media were prepared by mixing 1 volume of 10 $\times$ Dulbecco's modified Eagle's medium (Sigma) with 9 volumes of sterile deionized water and were supplemented with 0.584 g/L L-glutamine and 0.004 g/L folic acid (Sigma). Where required, starting pH was adjusted using 2 mol/L sodium hydroxide solution. pH was then measured over 24 or 48 hours using an Oakton pH Spear Waterproof Pocket pH Testr (Cole-Parmer, London, UK).

Figure 5 Cathepsin K and fibroblast-specific protein co-localize in lymphangioleiomyomatosis (LAM) lung tissue. **A:** Immunofluorescence staining in LAM lung tissue from three donors. Individual panels show DAPI staining of nuclei (blue), fibroblast surface protein (FSP; green), cathepsin K (CTSK; red), and the overlay with co-localization of FSP and CTSK (yellow). CTSK and FSP strongly co-localize, with only modest CTSK expression outside of LAM-associated fibroblasts. **B:** Dual chromogenic IHC analysis from the same three representative donors, showing LAM nodules reacting with antibodies against both CTSK (blue) and FSP (brown). All donors show spindle-shaped cells within nodules reacting with both antibodies. **Boxed area** correspond with the images to the right. Original magnification: $\times 20$ (**A**); $\times 4$ (**B**, left panels); $\times 40$ (**B**, right panels). Scale bars: 200 μm (**A**); 500 μm (**B**, left panels); 50 μm (**B**, right panels).

Membrane Transporters and Proton Pump Inhibitors

Inhibitors used were: CAs, S4 (IX and XII inhibitor; Tocris) 100 $\mu\text{mol/L}$ and acetazolamide (universal; Sigma) 1 mmol/L; sodium H^+ exchanger, BIX (Tocris) 100 nmol/L; sodium

HCO_3^- co-transporter, S0859 (Sigma) 50 $\mu\text{mol/L}$; vacuolar-type H^+ ATPase, concanamycin A (Santa Cruz, Insight Biotechnology, Middlesex, UK) 100 nmol/L; mTOR, rapamycin (Calbiochem, Merck Millipore, Watford, UK) 10 nmol/L.

Figure 6 Cathepsin K activity is pH dependent. **A:** 621-101 cells, lymphangioma-associated fibroblasts (LAFs), and 621-101–LAF co-cultures were grown in culture at a range of pH values. Cathepsin K activity is low at all pH values in 621-101 supernatants but increases at low pH in LAF cultures. Co-cultures have significantly higher cathepsin K activity than do 621-101 cell or LAF cultures at pH 7.0 and 6.0. **B:** 621-101 cells and LAFs were cultured in nonbuffered media for 24 hours at various starting pH values. LAFs do not significantly affect pH, whereas 621-101 cells acidify the media independent of starting pH. **C:** *TSC2*^{-/-} and *TSC2*^{+/+} murine embryonic fibroblasts (MEFs) were cultured in unbuffered media for 24 hours at pH 7.5. *TSC2*^{+/+} MEFs and *TSC2*^{-/-} MEFs and 621-101 cells treated with 10 nmol/L rapamycin do not affect pH, whereas untreated *TSC2*^{-/-} MEFs and 621-101 cells significantly acidify media. Data are expressed as means $\pm$ SD (**A** and **C**). *n* = 4 (**A**); *n* = 3 (**C**). ***P* < 0.01, ****P* < 0.001. RFU, relative fluorescence units.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software version 6 (GraphPad Software, Inc., La Jolla, CA). Paired experiments were analyzed by *t*-test and multiple comparisons by two-way analysis of variance with Dunnett or Bonferroni correction, with *P* values of <0.05 regarded as significant.

Results

Cathepsin K Is Overexpressed in LAM Lung Tissue

mRNA was extracted from whole-lung tissue of six patients with sporadic LAM. Normal human total lung RNA was obtained from Ambion (ThermoFisher Scientific, Paisley, UK). Quantitative RT-PCR was performed for candidate proteases and protease inhibitors MMP-1, -2, -3, -9, -13, and -14; tissue inhibitor of metalloproteinases 1, 2, and 3; cathepsins B, C, D, K, L, and S; urokinase-type plasminogen activator; urokinase-type plasminogen activator receptor; and plasminogen activator inhibitor 1. To determine that the method was appropriate, we first compared the expression of the LAM-specific genes premelanosome protein (*PMEL*), melan-A (*MLANA*), and vascular endothelial growth factor D (*VEGFD*) in control and LAM lungs. Transcripts for *PMEL*, *MLANA*, and *VEGFD* were 297-, 267-, and 2.47-fold more abundant in LAM than in control lung tissue (Supplemental Figure S1). mRNA was detected for all proteases examined. Transcript expression was variable between individual donors. The most strongly expressed protease transcript in LAM lung was cathepsin K, which was increased 40-fold compared with that in normal tissue (mean, 0.375 vs 0.00092; *P* $\leq$ 0.0001). Cathepsins B and D and tissue inhibitor of metalloproteinase 3 were significantly reduced in LAM; other proteases were unchanged (Figure 1A).

We next incubated fresh LAM lung tissue in culture with rapamycin, estrogen, or vehicle for 48 hours, with cathepsin K expression measured by quantitative RT-PCR. Six tissue explants obtained from different areas of the lung parenchyma of two donors were assessed. Cathepsin K gene expression was detected in all tissue explants. Rapamycin (10 nmol/L) reduced cathepsin K gene expression to around one quarter of the levels in vehicle-treated controls (*P* < 0.001). Estrogen (10 nmol/L) had no significant effect on cathepsin K gene levels (Figure 1B).

Cathepsin K protein was examined using IHC analysis in six lung biopsy samples and seven transplant tissue samples from women with LAM. LAM nodules were identified using immunostaining for α -smooth muscle actin, the melanoma marker PNL2, and fibroblast surface protein. LAM nodules were located adjacent to lung cysts (Figure 2). Cathepsin K was expressed within LAM nodules in all cases but was not present in surrounding noninvolved lung tissue from patients with LAM or from control patients (Figure 2).

Figure 7 Expression of membrane transporters in lymphangioleiomyomatosis (LAM) cells and lung tissue. **A:** Real-time quantitative PCR for carbonic anhydrase (CA) II, IX, and XII; monocarboxylate transporters 1 and 4; sodium bicarbonate ($\text{Na}^+/\text{HCO}_3^-$) co-transporters 1/*SLC4A4* and 3/*SLC4A7*; sodium H^+ (Na^+/H^+) exchanger (NHE) 1/*SLC9A1*; and vacuolar-type H^+ -ATPases (V-ATPases) *ATP6V1B2* and *ATP6V0A4* in 621-101 cells treated for 24 hours with either vehicle, 10 nmol/L rapamycin, 10 nmol/L estrogen, or LAM-associated fibroblast-conditioned medium (LAF-CM). **B:** Real-time quantitative PCR for CA II, IX, and XII; monocarboxylate transporters 1 and 4; Na^+/H^+ exchanger; NHE1; $\text{Na}^+/\text{HCO}_3^-$ co-transporters *SLC4A4* and *SLC4A7*; and V-ATPases *ATP6V1B2* and *ATP6V0A4* in six LAM- and three control patient-derived lung tissues. **C:** IHC staining of two representative LAM lung tissues, showing positive staining in serial sections for CA IX and *SLC4A4* within LAM nodules. Normal lung shows positive staining for *SLC4A4* but not CA IX. **D:** Pharmacologic inhibition of membrane transporters or mTOR inhibits extracellular acidification in 621-101 cell cultures. S4 and acetazolamide (carbonic anhydrase inhibitors), BIX (Na^+/H^+ exchanger inhibitor), S0859 ($\text{Na}^+/\text{HCO}_3^-$ co-transporter inhibitor), and concanamycin A (V-ATPase inhibitor). Data are expressed as means $\pm$ SD (**A** and **B**). $n = 3$. Scale bars: 50 μm (**C**, first and third columns); 500 μm (**C**, second and fourth columns). Original magnification: $\times 4$ (**C**, first and third columns); $\times 40$ (**C**, second and fourth columns).

Cathepsin K expression was particularly strong in the spindle-like cells within nodules that were also immunopositive for fibroblast surface protein and α -smooth muscle actin (Figure 3).

Association of Cathepsin K with Fibroblast-Like Cells in LAM Nodules

LAM nodules are heterogeneous structures with a complex mixture of cell types. To determine which cell types are involved in cathepsin K expression we first examined the expression of the *CTSK* transcript by RT-PCR in normal human lung fibroblasts, LAFs, and 621-101 cells. *CTSK* transcript was present in both normal lung fibroblasts and LAF but was not significantly expressed by 621-101 cells (Figure 4A).

On immunofluorescence, normal human lung fibroblasts and LAF, but not 621-101 cells, were positive for cathepsin

K protein, which was concentrated in intracytoplasmic granules (Figure 4B). To determine the presence of intracellular cathepsin activity, we then used Magic Red, a substrate that generates red fluorescence when processed by cathepsins. In LAFs, Magic Red fluorescence was detected and was partially inhibited by the cathepsin K inhibitor L006235 and completely inhibited by the broad-spectrum cysteine protease inhibitor E64 (Figure 4C).

To determine whether LAFs are the predominant source of cathepsin K in LAM lung tissue, we co-immunostained using differential immunostaining with both chromogenic and fluorescent labels. Using both systems we observed strong co-localization of cathepsin K and fibroblast surface protein in LAM nodules consistent with expression of cathepsin K by LAM lung fibroblasts. A lower level of cathepsin K staining could also be detected by immunofluorescence in fibroblast surface protein negative cells (Figure 5).

Figure 8 Inhibition of membrane transporters blocks extracellular acidification and cathepsin K activity in lymphangioleiomyomatosis (LAM) cell co-cultures. **A:** 621-101–LAM-associated fibroblast co-cultures were grown in unbuffered media in the presence of either vehicle control or S4 (carbonic anhydrase IX, XII inhibitor), acetazolamide (nonspecific carbonic anhydrase inhibitor), BIX (Na⁺/H⁺ exchanger inhibitor), S0895 (Na⁺/HCO₃⁻ co-transporter inhibitor), concanamycin A (V-ATPase inhibitor), or rapamycin [mechanistic target of rapamycin (mTOR) inhibitor]. **B:** Cathepsin K activity in co-culture supernatants treated with inhibitors of H⁺ ion transporters showing strong inhibition of both acidification and cathepsin K activity by inhibition of the Na⁺/HCO₃⁻ co-transporter and mTOR. Data are expressed as means ± SD (**B**). *n* = 4. ***P* < 0.01, ****P* < 0.001. CA, carbonic anhydrase; RFU, relative fluorescence units.

Cathepsin K Activity Is pH Dependent

We examined cathepsin K activity *in vitro* using four separate LAF primary cultures and 621-101 cells both separately as monocultures and combined in co-cultures. At physiological pH, cathepsin K activity was not significantly elevated above baseline values in any cell type or culture condition. As cathepsin K requires low pH for its activity, cell cultures were also studied at pH 7.0 and 6.0. Cell viability was unimpaired at pH 6 and above over 24 hours, as assessed by MTT reduction (Supplemental Figure S2). At pH 7.0 and 6.0, LAF cathepsin K activity was 1.7- and 2.2-fold higher than at pH 7.5 (*P* = 0.044 and 0.0017,

respectively), and almost threefold higher in co-cultures (*P* ≤ 0.0001). Cathepsin K activity in 621-101 cell supernatants was low at all pH values (Figure 6A).

TSC2^{-/-} Cells Acidify the Extracellular pH as a Consequence of mTOR Dysregulation

As LAF-derived cathepsin K requires low pH for its proteolytic activity, we set out to determine whether cells within LAM nodules could acidify tissue culture medium *in vitro*. 621-101 cells and LAFs were grown in unbuffered tissue culture medium at initial pH values of 7.5, 7.0, and 6.0. LAFs had no significant effect on culture medium pH over 24 hours. 621-101 cell culture medium fell by around half of 1 pH unit over 24 hours independent of the starting pH value (Figure 6B).

To determine whether extracellular acidification was a consequence of mTOR dysregulation, we examined MEFs lacking *TSC2*, a negative regulator of mTORs and their genotypic *TSC2*^{+/+} counterparts. MEFs and 621-101 cells were grown in unbuffered tissue culture medium at initial pH values of 7.5, 7.0, and 6.0. Cell viability of both *TSC2*^{+/+} and *TSC2*^{-/-} MEFs was unimpaired at low pH over 24 hours (Supplemental Figure S3). *TSC2*^{+/+} MEFs had no significant effect on extracellular pH over 24 hours. *TSC2*^{-/-} MEFs and 621-101 cells reduced the culture medium pH by 0.35 and 0.51 pH units from a starting pH of 7.5 (*P* = 0.0067 and *P* = 0.0004, respectively) (Figure 6C). Treatment of *TSC2*^{-/-} MEFs and 621-101 cells with rapamycin completely abrogated the change in pH over 24 hours. Similar findings were observed at starting pH values of 7.0 and 6.0 (data not shown).

Expression of H⁺ Ion Transporters in LAM

To determine the mechanism of extracellular acidification by 621-101 cells we profiled candidate membrane transporter expression in 621-101 cells using real-time quantitative PCR. CAs II, IX, and XII; monocarboxylate transporters 1 and 4, sodium bicarbonate (Na⁺/HCO₃⁻) cotransporters 1/*SLC4A4* and 3/*SLC4A7*; sodium H⁺ (Na⁺/H⁺) exchanger; sodium H⁺ (Na⁺/H⁺) exchanger member 1/*SLC9A1*; and vacuolar-type H⁺-ATPases *ATP6V1B2* and *ATP6V0A4* were all expressed in 621-101 cells (Figure 7A). When 621-101 cells were incubated with rapamycin, estrogen- or LAF-conditioned medium, CA IX gene expression was reduced in the presence of rapamycin, although no other changes were significant. In LAM and control lung tissue, gene expression levels of CAs II and XII; monocarboxylate transporters 1 and 4; sodium H⁺ (Na⁺/H⁺) exchanger 1; *SLC4A4* and *SLC4A7*; and *ATP6V1B2* and *ATP6V0A4* were similar (Figure 7B). We then examined the expression of the two most strongly expressed transporter proteins, CA IX and the Na⁺/HCO₃⁻ co-transporter *SLC4A4*, in lung tissue. Both

Figure 9 Proposed mechanism of cathepsin K activation in lymphangioleiomyomatosis (LAM). *TSC2*^{-/-} LAM cells express carbonic anhydrases (CA) II, IX, and XII within the cell, which, in tandem, catalyze the conversion of CO₂ and water to H⁺ and HCO₃⁻ ions. Na⁺/HCO₃⁻ co-transporters (NBC) shuttle HCO₃⁻ ions into the cell, resulting in net H⁺ export into the extracellular space. Cathepsin K-containing lysosomes of LAM-associated fibroblasts translocate to the cell membrane discharging cathepsin K, where it is activated in the low extracellular pH. ECM, extracellular matrix; mTOR, mechanistic target of rapamycin.

CA IX and SLC4A4 were strongly expressed in LAM nodules. SLC4A4, but not CA IX, was present in control lung tissue (Figure 7C).

Inhibition of Membrane Transporters Affects 621-101 Cell Extracellular pH and Cathepsin K Activity

We then used pharmacologic inhibitors of these membrane transporters to determine whether we could inhibit extracellular acidification by 621-101 cells. In unbuffered media treated with vehicle control, 621-101 cells reduced extracellular pH by 0.75 pH units over 24 hours. Inhibition of vacuolar-type H⁺-ATPases, CAs, Na⁺/H⁺ exchanger, and the Na⁺/HCO₃⁻ co-transporter blocked extracellular acidification increasingly strongly. Interestingly the mTOR inhibitor rapamycin was more potent than any of the membrane transporter inhibitors and completely abolished extracellular acidification (Figure 7D).

To recapitulate the LAM nodule environment, we next examined whether 621-101 cells were capable of acidifying their environment in the presence of LAF and whether this resulted in activation of LAF-derived cathepsin K. 621-101–LAF co-cultures acidified the extracellular space, which was associated with cathepsin K activity in the co-cultures (Figure 8). Membrane transporter inhibitors blocked extracellular acidification to the same degree as seen in 621-101 monocultures. Inhibition of pH change was also associated with reduced cathepsin K activity. Importantly, the Na⁺/HCO₃⁻ co-transporter inhibitor was the strongest inhibitor of both acidification and cathepsin K activity, reducing activity by almost 75%. Inhibitors with more modest effects on acidification had had lesser effects on cathepsin K activity. Again, rapamycin was the strongest inhibitor of acidification and reduced cathepsin K activity

by around 50%. The inhibitors used did not affect cell viability (Supplemental Figure S4).

Discussion

Here we have shown that cathepsin K expression in LAM is mainly dependent on the presence of fibroblasts within LAM nodules. *In vitro*, LAF-derived cathepsin K is only active at pH <7.0 and importantly, LAM-derived 621-101 cells, in common with other *TSC2*^{-/-} cell lines, express net hydrogen ion exporters that acidify their local environment to the extent that cathepsin K is activated. Our findings show that cell–cell interactions within the LAM nodule stroma can generate the conditions in which proteolytic lung damage may occur.

Cathepsin K has a primary role as a bone-remodeling protease expressed by osteoclasts and dependent for its extracellular activity on the low pH in bone-resorbing lacunae generated by CAs, vacuolar-type H⁺-ATPases, Na⁺/H⁺ exchangers, and chloride bicarbonate exchangers.^{24,32} Cathepsin K is a potent collagenase and elastase, but also selectively processes ELR chemokines, which enhances their chemotactic activity,³³ suggesting a potential role in inflammatory cell chemotaxis. Unlike the metalloproteinases and serine proteases previously described in LAM, cathepsin K is not present in normal lung tissue but is expressed strongly by tumor stromal fibroblasts.²⁵ Expression of cathepsin K in LAM and other perivascular epithelioid cell tumors was first described by Chilosi et al,²² who also suggested that cathepsin K expression may be mTOR dependent. Here we show that by suppressing mTOR activity in LAM lung tissue with rapamycin, cathepsin K gene expression was significantly reduced. In the osteoclast, cathepsin K expression is

dependent on microphthalmia transcription factor, a helix-loop-helix transcription factor that regulates melanocyte development, cyclin-dependent kinase, and anti-apoptotic gene expression.³⁴ Microphthalmia transcription factor binds three consensus sites in the cathepsin K promoter as a heterodimer with various partners, including transcription factor E3, and is partially mTOR dependent.³⁴ Moreover, mTOR inhibition in human osteoclasts reduced cathepsin K protein expression and bone resorption,³⁵ raising the possibility that the inhibition of mTOR and cathepsin K may have synergistic effects on the inhibition of lung destruction in LAM.

The requirement for low pH to activate cathepsin K is well described.^{36,37} Monocyte-derived macrophages acidify their pericellular environment via vacuolar-type H⁺-ATPases, thus enabling them to maintain cathepsin K in its active form.³⁸ Here we have shown that acidic conditions may exist within a LAM nodule and that this extracellular acidification is a consequence of mTOR dysregulation, likely to result both in the expression of membrane transporters (including, CAs, monocarboxylate transporters, and Na⁺/HCO₃⁻ co-transporters) and in mTOR dysregulation (causing the Warburg effect, a metabolic dependence on aerobic glycolysis) (Figure 9). In 621-101 cell–LAF co-cultures, the transporters acidify the extracellular space, resulting in the activation of cathepsin K, whereas their inhibition, particularly of the Na⁺/HCO₃⁻ co-transporters, blocks both extracellular acidification and protease activation. Strikingly, rapamycin completely and rapidly abrogated acidification in culture despite only suppressing the transcription of CA IX, suggesting that part of the effect may have been on 621-101 cell metabolism rather than exclusively on the transporters themselves.

Inhibition of the mTOR pathway is the only proven treatment for LAM but does not arrest lung destruction in all cases.³⁹ Our findings suggest that part of the beneficial effect of mTOR inhibition may be the suppression of Warburg metabolism and extracellular acidification, a phenomenon also observed in lymphoma models.⁴⁰ Small-molecule inhibitors of CAs and sodium bicarbonate cotransporters have been successfully used in preclinical cancer models^{41–44} and may have synergistic benefits with mTOR inhibition in LAM to reduce destructive protease activation. In addition, direct inhibition of cathepsin K has been shown to reduce bone loss in osteoporosis.⁴⁵ Combinations of these therapeutic approaches may be superior to mTOR inhibition alone in reducing lung destruction in LAM.

Although the 621-101 cells and LAFs in culture may not completely recapitulate the LAM nodule environment, we have been careful to show that, in addition to the findings from our *in vitro* studies, the elements necessary to synthesize and activate cathepsin K are present in human lung tissue. Although we have not directly shown that there is an acidic environment in human LAM lung nodules, the

presence of these components and the existence of an analogous situation in human cancers suggest that this is likely to be the case.

Taken together, our findings suggest that LAM cell–LAF interactions within LAM nodules promote disease progression by protease activation in a manner similar to tumor cell–cancer-associated fibroblast interactions in cancer. These similarities are consistent with the idea that LAM is a low-grade neoplastic disease, with a stroma similar to cancer.⁴⁶ Moreover, mTOR inhibitors that reduce lung function decline in LAM,^{39,47,48} may exert some of their protective action in the lung on LAM cell–related extracellular pH, cathepsin K activation, and expression. The expression of transporters, including the Na⁺/HCO₃⁻ co-transporter, is downstream of mTORC1/hypoxia-inducible factor 1 α .^{49,50} Further studies are required for understanding how mTOR activation and the expression of membrane transporters are related and whether the inhibition of these transporters or cathepsin K activity will be of benefit in addition to mTOR inhibitors for LAM.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.04.014>.

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