The American Journal of Pathology, Vol. ■, No. ■, ■ 2017

ELSEVIER

The American Journal of PATHOLOGY

ajp.amjpathol.org

Cathepsin K in Lymphangioleiomyomatosis

LAM Cell—Fibroblast Interactions Enhance Protease Activity by Extracellular Acidification

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Accepted for publication April 26, 2017.

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Q4 Lymphangioleiomyomatosis (LAM) is a rare disease in which clonal LAM cells infiltrate the lungs and lymphatics. In association with recruited fibroblasts, LAM cells form nodules adjacent to lung cysts. It is hypothesized that LAM nodule–derived proteases lead to cyst formation. On protease gene–expression profiling in whole-lung tissue, cathepsin K was 40-fold overexpressed in LAM compared with control lungs ($P \leq 0.0001$). Immunohistochemistry confirmed cathepsin K protein in LAM nodules but not in Q5 control lungs. Cathepsin K gene expression and protein and protease activity were detected in LAM-associated fibroblasts but not in LAM cell line 621-101. In lung nodules, cathepsin K immunoreactivity predominantly co-localized with LAM-associated fibroblasts. *In vitro*, extracellular cathepsin K activity was minimal at pH 7.5 but was significantly enhanced in fibroblast cultures at pH 7 and 6. 621-101 cells reduced extracellular pH by 0.5 units over 24 hours. Acidification was dependent on 621-101 mechanistic target of rapamycin activity and net hydrogen ion transporters, 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 inhibited by sodium bicarbonate co-transporter ($P \leq 0.0001$) and carbonic anhydrase inhibitor ($P = 0.0021$). Our findings suggest that cathepsin K activity is dependent on LAM cell—fibroblast interactions; inhibitors of extracellular acidification may be potential therapies for LAM. *(Am J Pathol 2017, ■: 1–13; http://dx.doi.org/10.1016/j.ajpath.2017.04.014)*

Q6 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 tuberin, 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, Q7 including α-smooth muscle actin, and melanocyte lineages with microphthalmia transcription factor, glycoprotein 100, and PNL2.[7] This mixed phenotype is characteristic of the Q8 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 cells[9] including fibroblasts,[10] mast cells,[11] and other inflammatory cells.[12]

Supported by LAM Action grant LAM002 and University of Nottingham postgraduate studentship award 072. Q2

Disclosures: The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Ethical approval for the use of LAM lung tissue was given by the University of Nottingham Research Ethics Committee, and written, informed consent was obtained from all patients.

http://dx.doi.org/10.1016/j.ajpath.2017.04.014

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 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 protease[24] 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 obtained 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 tuberin 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 Lisa Henske (Harvard University, 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 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-μm pore size; Corning Life Sciences, SLS, Nottingham, UK) were equilibrated for 1 hour at 37°C and 5% CO_2 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 μL 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.

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