CHEST

Original Research

DIFFUSE LUNG DISEASE

Sirolimus Decreases Circulating Lymphangioleiomyomatosis Cells in Patients With Lymphangioleiomyomatosis

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Background: **Lymphangioleiomyomatosis (LAM), sporadic or in women with tuberous sclerosis complex (TSC), is characterized by cystic lung destruction, lymphatic involvement (eg, chylous pleural effusions, lymphangioleiomyomas), and renal angiomyolipomas (AMLs). The multisystem manifestations of LAM appear to result from metastatic dissemination of LAM cells bearing inactivating mutations or having loss of heterozygosity (LOH) of the tumor suppressor genes *TSC1* or *TSC2*, which leads to hyperactivation of the mammalian target of rapamycin. Sirolimus slows the decline of lung function, reduces chylous effusions, and shrinks the size of AMLs. The purpose of this study was to determine the effect of sirolimus on circulating LAM cells.**

Methods: **Cells from blood were isolated by a density-gradient fractionation system and from urine and chylous effusions by centrifugation. Blood cells were incubated with anti-CD45-fluorescein isothiocyanate (FITC) and anti-CD235a-R-phycoerythrin (PE) antibodies, and urine and chylous effusion cells were incubated with anti-CD44v6-FITC and anti-CD9-R-PE antibodies. Cells were sorted and analyzed for *TSC2* LOH.**

Results: **LAM cells with *TSC2* LOH were identified in 100% of blood specimens and 75% of urine samples from patients before therapy. Over a mean duration of 2.2 ± 0.4 years of sirolimus therapy, detection rates of LAM cells were significantly decreased to 25% in blood ($P < .001$) and 8% in urine ($P = .003$). Following therapy, a greater loss of circulating LAM cells was seen in postmenopausal patients ($P = .025$).**

Conclusions: **Patients receiving sirolimus had a progressive loss of circulating LAM cells that depended on time of treatment and menopausal status.** ***CHEST 2014; 145(1):108–112***

Abbreviations: AML = angiomyolipoma; LAM = lymphangioleiomyomatosis; LOH = loss of heterozygosity; mTOR = mammalian target of rapamycin; NIH = National Institutes of Health; PCR = polymerase chain reaction; TSC = tuberous sclerosis complex

Lymphangioleiomyomatosis (LAM), a rare multisystem disorder affecting primarily women of child-bearing age, is characterized by cystic lung destruction, lymphatic involvement (eg, chylous pleural effusions, lymphangioleiomyomas), and renal angiomyolipomas (AMLs).[1] LAM occurs sporadically or in association with tuberous sclerosis complex (TSC), an autosomal dominant disorder. The multisystem manifestations of LAM are believed to result from metastatic dissemination of abnormal smooth muscle-like LAM cells bearing inactivating mutations or having loss of heterozygosity (LOH) of one of the two tumor suppressor genes *TSC1* and *TSC2*,[2] which leads to hyperactivation of mammalian target of rapamcyin (mTOR).[3,4] mTOR controls cell proliferation, growth, and motility.[5] Because mutations in genes encoding proteins upstream of mTOR and regulatory abnormalities in critical cellular pathways lead to hyperactivation of mTOR, rapamycin (sirolimus) and its analogs are being used to treat cancers (eg, renal cell carcinoma), hamartomas (eg, AMLs), vascular diseases (eg, antirestenosis following angioplasty), and allograft rejection (eg, immunosuppression).[6-8] In addition, rapamycin has been used to target metastatic processes in animals models.[9,10]

Patients treated with sirolimus have shown improvement in lung function,[8] reduction of chylous effusions,[11] and decrease in the size of AMLs.[7] Although

sirolimus blocks proliferation by arresting the cell cycle,[12] effects of sirolimus on circulating cells, which may be critical to metastasis and tumor dissemination, have not been demonstrated in human disease. Here, we studied the effect of sirolimus on circulating LAM cells found in patients with LAM.[13,14] To determine the effect of sirolimus on circulating LAM cells, we investigated the presence of LAM cells in the blood, chylous effusions, and urine in patients receiving and not receiving sirolimus therapy.

Materials and Methods

Study Design

Twenty-three patients with LAM were enrolled between 2007 and 2012 at the National Institutes of Health (NIH) Clinical Center in clinical protocols (95-H-0186; 96-H-0100) approved by the National Heart, Lung, and Blood Institute Institutional Review Board. The diagnosis of LAM was based on clinical, radiographic, and histopathologic findings. Patients with only high-resolution CT scan-compatible cystic disease were not judged to have LAM, unless a biopsy specimen was obtained or the patients had consistent clinical findings (eg, TSC, extrapulmonary manifestations [AMLs, lymphangioleiomyomas, chylous pleural effusions, or ascites]). Local physicians prescribed sirolimus and adjusted the dose to maintain serum levels between 5 and 15 ng/mL. Samples of blood, urine, and chylous effusions were collected from patients before and after initiation of sirolimus.

Enrichment and Isolation of Circulating LAM Cells by Fluorescence-Activated Cell Sorting

Blood (50-75 mL) was fractionated by density-gradient centrifugation on OncoQuick columns (Greiner Bio-One) as previously described.[14] Urine (500 mL) from a 24-h collection and chylous effusions (100 mL) were centrifuged (300*g*, 10 min, 4°C). Enriched cells from blood were incubated for 30 min at room temperature with anti-CD45-fluorescein isothiocyanate (clone HI30) and anti-CD235a-R-phycoerythrin (clone GA-R2) antibodies (BD Biosciences). Cells from urine and chylous effusions were reacted with anti-CD44v6-fluorescein isothiocyanate (clone VFF-7) (Invitrogen, Life Technologies Corporation) and anti-CD9-R-phycoerythrin (M-L13) antibodies (BD Biosciences). After incubation, cells were washed once in phosphate-buffered saline and analyzed and sorted as previously described.[14]

Polymerase Chain Reaction Analysis of LOH

Genomic DNA was isolated from whole blood and unsorted or sorted cells with the use of the QIAamp DNA Micro Kit (QIAGEN) and amplified at loci D16S291, Kg8, D16S3395, D16S3024, and D16S521 on chromosome 16p13.3 as previously described.[13,14] Polymerase chain reaction (PCR) was performed in 10-μL mixtures containing 1 × PCR Gold Buffer, 1.5 mmol/L $MgCl_2$, 250 μmol/L each deoxynucleoside triphosphate, 0.8 μmol/L primers, and 2 units AmpliTaq Gold DNA polymerase (Applied Biosystems, Life Technologies Corporation) with a Veriti 96-Well Thermal Cycler (Applied Biosystems) with initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C (30 s), 55°C (45 s), and 72°C (45 s) and a final extension at 72°C for 2 min. PCR products were analyzed on a 3100 Genetic Analyzer (Applied Biosystems). Q^{LOH} was determined by comparing the ratio of fluorescence intensities of each allele in putative LAM (L) cells to that in whole blood (N) from the same patient with (L1/L2)/(N1/N2), where L1 or N1 is the minor allele and L2 or N2 the other. Q^{LOH} values < 0.5 or > 0.62 were scored as LOH or retention of heterozygosity, respectively, whereas no definite decision was made with Q^{LOH} values 0.5 to 0.62.

Statistical Analysis

We used generalized linear models with repeated measures to compare the binary outcome variable of detection of *TSC2* LOH before and after sirolimus therapy and between blood and urine samples. A first-order autoregressive structure was used to model the correlations in the repeated measurements. We performed a multivariate analysis where the combined data of both fluid tests were used along with treatment time and menopausal status to determine factors associated with detection of LAM cells. ORs and 95% CIs were derived. However, the comparison of detection of *TSC2* LOH before and after sirolimus therapy could not be performed with blood samples because the detection rate of LOH before sirolimus therapy in these patients was 100%, so no estimates could be derived. We compared differences in detection rates of *TSC2* LOH before and after sirolimus therapy with Fisher exact test. Continuous data are reported as mean ± SEM. Two-tailed statistical tests were used, and $P < .05$ was considered significant. All statistical analyses were performed with the SPSS version 15.0 for Windows (IBM Corporation) software.

Results

Twenty-three patients with LAM who fit the study inclusion criteria were enrolled between 2007 and 2012 at the NIH Clinical Center. Baseline demographic and clinical characteristics are shown in Table 1. Samples of blood, urine, and chylous effusions were collected at NIH before and during sirolimus therapy. Sirolimus was prescribed by local physicians and the dose adjusted to maintain serum levels between 5 and 15 ng/mL. Cells from blood, chylous effusions, and urine were sorted on the basis of cell surface markers (CD235a, CD45, CD44v6, CD9) that have been

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