CHEST Online Supplement

Sirolimus Decreases Circulating Lymphangioleiomyomatosis Cells in Patients With Lymphangioleiomyomatosis

Xiong Cai, PhD; Gustavo Pacheco-Rodriguez, PhD; Mary Haughey, RN, BSN; Leigh Samsel, MS; Suowen Xu, PhD; Hai-Ping Wu, BS; J. Philip McCoy, PhD; Mario Stylianou, PhD; Thomas N. Darling, MD, PhD; and Joel Moss, MD, PhD, FCCP

e-Appendix 1.

To determine the effect of sirolimus on the expression of cell-surface markers in cultured cells from LAM lungs, the ability of LAM cells migrating to the layer of cells obtained during the Oncoquick separation after sirolimus treatment, and the effect of long treatment with sirolimus on the expression of receptors, we carried out the following experiments.

First, we grew LAM cell line 1 and human *TSC2*$^{-/-}$ skin fibroblast as described [1,2] _ENREF_1_ENREF_1. After treatment with 200 nM sirolimus or vehicle for 3 days, cells were labeled for flow cytometric analysis with antibodies against CD45, CD235a, CD44v6, and CD9. While 40 to 50% of blood cells are positive for CD45, no appreciable CD45+ cells were seen in both LAM cell line 1 and *TSC2*$^{-/-}$ cells. Reactivity (panels A and B) and mean fluorescence intensity (MFI) (panels C and D) of LAM cell line 1 and *TSC2*$^{-/-}$ cells to CD235a, CD44v6, and CD9 were similar in sirolimus-treated cells as in vehicle-treated cells. Thus, it appears that the cell surface proteins used for LAM cell isolation are not affected by sirolimus.

Second, We wondered whether sirolimus treatment of LAM cells could affect cell density so that LAM cells would not migrate to the same layer of tumor cells in the Oncoquick procedure. For this reason, we grew LAM line 1 as described [2], and treated with 200 nM sirolimus or vehicle for 10 days. Then, 25 ml of blood samples from healthy volunteers were mixed with 1×10^6 LAM cells, the cell mixture was subjected to Oncoquick separation. Cell fractions were separated by the OncoQuick density-gradient procedure and were sorted following incubation with anti-CD45 and anti-CD235a antibodies. As shown in the figure below, PCR microsatellite analysis of informative microsatellite markers D16S3395 for the cell line (LAM line 1) (~96 kb telomeric to *TSC2*) revealed genotypes

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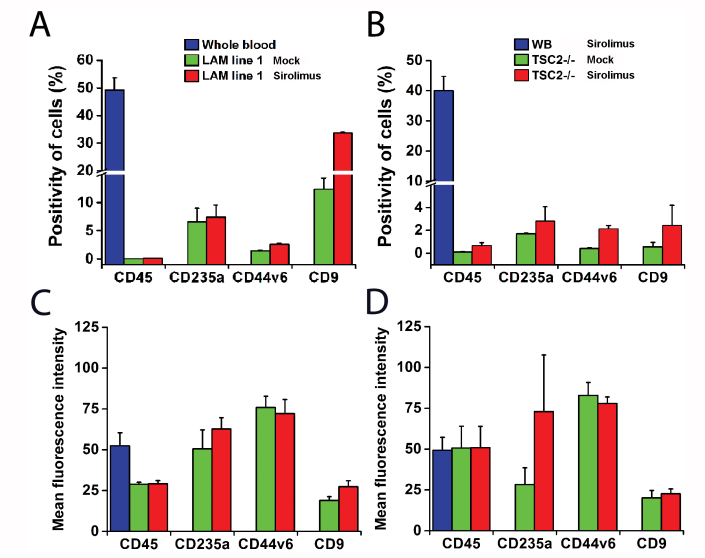
consistent with the presence of cells from LAM line 1 cells in both treated with sirolimus (panel B) or vehicle (panel A). These data suggest that sirolimus treatment does not affect the ability of LAM cells to migrate to the LAM cell layer in the Oncoquick separation.

Third, to evaluate long-term effect of sirolimus on LAM cells, we grew LAM cell line 1 in the presence of 200 nM sirolimus or vehicle for 40 days, and cells were incubated with anti-CD44v6 and CD9. Cells were sorted as indicated below (e-Figure 3). LAM cells were screened using the informative microsatellite marker D16S3395. *TSC2* LOH was consistently detected in $CD44v6^{+}CD9^{+}$ cell population without or with sirolimus treatment. Although the population $CD45^{-}CD235a^{+}$ showed a Q^{LOH} between 0.5 and 0.6 indicating allelic imbalance, we were not able to determine loss of heterozygosity in these cells.



e-Figure 1. Status and expression levels of cell surface molecules CD45, CD235a, CD44v6, and CD9 on LAM lung-derived cell line 1 and *TSC2*$^{-/-}$ skin tumor cells treated with sirolimus. (*A*) and (*C*) show reactivity and mean fluorescence intensity (MFI) of LAM line 1 and (*B*) and (*D*) show reactivity and MFI of *TSC2*$^{-/-}$ cells as assessed by flow cytometric analysis. Results are presented as means ±SEM. Experiments were replicated three times.

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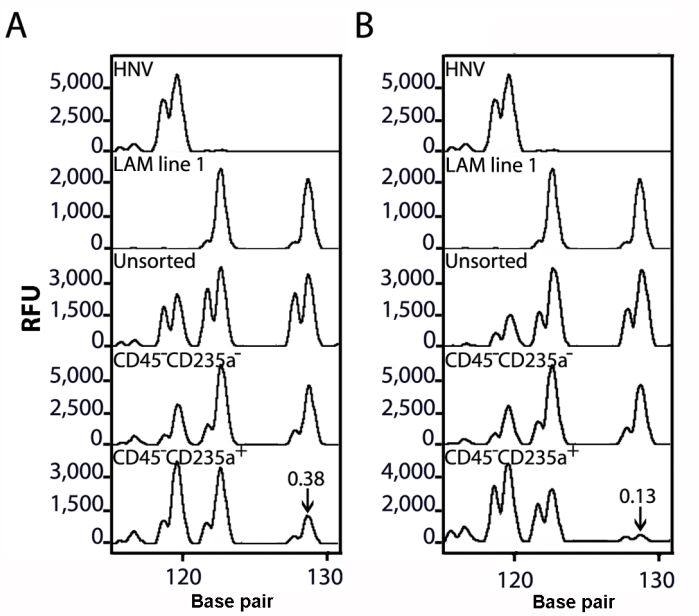
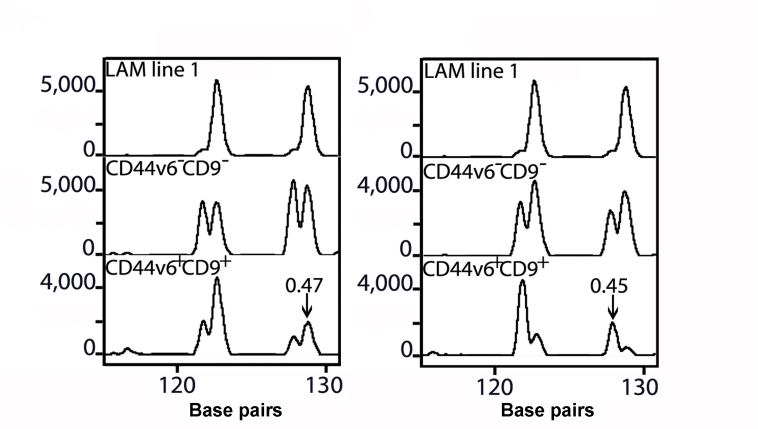


e-Figure 2. Detection of cultured LAM lung cells treated sirolimus in blood from a healthy volunter. LAM lung-derived cell line 1 was grown and treated with 200 nM sirolimus or vehicle for 10 days. 25 ml of blood samples from healthy volunteers were mixed with 1×10^6 LAM line 1, and then separated by the OncoQuick density-gradient procedure. Low-density cell fractions were further sorted with anti-CD45 and anti-CD235a antibodies. PCR analysis of informative microsatellite markers D16S3395 revealed genotypes consistent with the presence of LAM line 1 treated with vehicle (*A*) and rapamycin (*B*) in blood cells from healthy volunteers. *TSC2* LOH was detected in $CD45^-CD235a^+$ cell population from both LAM line 1 treated with vehicle (*A*) or rapamycin (*B*). Experiments were replicated three times. Experiments were replicated three times. **RFU, Relative Fluorescence Units.**



e-Figure 3. Detection of *TSC2* LOH in LAM cell line 1 treated with sirolimus for 40 days. PCR analysis of informative microsatellite marker D16S3395 showed LOH in $CD44v6^+CD9^+$ cell population (lowest panel) in cells treated with vehicle (*A*) and sirolimus (*B*) LAM line 1. Experiments were replicated three times. **RFU, Relative Fluorescence Units.**

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