Erythropoietin-driven proliferation of cells with mutations in the tumor suppressor gene TSC2

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LAM is characterized by airflow obstruction and impairment of gas exchange; cardiopulmonary exercise tests demonstrate the occurrence of hypoxemia during exercise, even in patients with near-normal lung function (48). These episodes of hypoxemia may not be recognized by the patient. Consequently, during activities of daily living, patients with LAM may experience recurrent hypoxemia.

Cells respond to hypoxic conditions by rapid synthesis and stabilization of hypoxia-inducible transcription factors (HIF-1α and HIF-2α), which heterodimerize with the constitutively expressed protein HIF-1β, resulting in transcriptional regulation of genes involved in erythropoiesis, glycolysis, cellular growth, apoptosis, and angiogenic signaling (40). Among the gene products induced by HIFs during chronic hypoxia is erythropoietin (EPO), which promotes survival and differentiation of erythropoietic cells (38), as well as proliferation of cells of nonerythropoietic origin, including smooth muscle cells (24, 33). EPO also enhanced indirectly the formation of smooth muscle-rich vascular lesions (25). Effects of EPO on nonerythroid cells have been correlated with cellular migration (1, 31) and increased tumor growth (30), including squamous tumors of head-neck, neuroblastomas (43), and lung cancer cells (27, 43).

Because hypoxemia may be frequent in LAM (48), we hypothesized that it could be associated with an increase in red cell indexes, presumably resulting from increased EPO production. Since LAM cells exhibit a smooth-muscle cell phenotype, we questioned whether an increase in EPO production would affect LAM cell proliferation and have an adverse effect on disease progression. The first objective of our study was to determine the relationships between red blood cell (RBC) indexes, severity of lung disease, and rate of decline in lung function. The second objective was to define the mechanisms whereby EPO could promote the growth of lung LAM lesions and LAM cells. We hypothesized that chronic, recurrent hypoxia in LAM contributes to the activation of an EPO-dependent signal transduction pathway in LAM cells, resulting in LAM cell proliferation, thereby hastening decline in lung function.

MATERIALS AND METHODS

More details can be found in the supplemental material. (The online version of this article contains supplemental data.)

Study population. The study population comprised 277 patients with LAM referred to the National Institutes of Health (NIH) for participation in a natural history study [National Heart, Lung, and Blood Institute (NHLBI) Protocol 95-H-0186], which was approved by the Institutional Review Board of the NHLBI. All subjects gave
informed consent before enrollment. The tissue sections from patients with idiopathic pulmonary fibrosis were obtained under NHLBI Protocol 99-H-0068.

Pulmonary function tests. Lung volumes, forced expiratory volume in the first second (FEV₁), and lung diffusion capacity (DLCO) were measured using a computerized system (Master Screen PFT, Erich Jaeger, Wuerzburg, Germany), according to American Thoracic Society standards (3–5).

Cardiopulmonary exercise testing. Patients were exercised on a bicycle ergometer or treadmill using a computerized metabolic cart (Vmax 229 Cardiopulmonary Exercise System; Sensormedics, Yorba Linda, CA), according to standard incremental protocols (48). Peak oxygen uptake was defined as the maximal value reached in any 30-s period (48).

Arterial blood gases. Arterial blood samples were obtained by radial artery puncture with the patient at rest, in the sitting position, breathing room air. Additional information is in the supplemental material.

Serum EPO, RBC, and hemoglobin levels. Serum EPO assays were performed in a clinical laboratory on blood samples collected in the morning from patients in the fasting state. Normal values were 5.0 to 24.6 mU/ml. Normal range of RBC (women, 3.58–4.99) and hemoglobin (11.1–15.0) levels were established at the Hematology Department of the NIH Clinical Center using blood from healthy blood donors collected at the NIH blood bank. These ranges were established by using a central 95% in a Celldyn Abbott Saphire, 4000, 3500 CBC analyzers.

LAM histology score. LAM histology score (LHS) of disease severity, based on the extent of replacement of lung tissue by cystic lesions and infiltration by LAM cells in open lung biopsy specimens, was quantified as follows: LHS-1 < 25%; LHS-2 = 25–50%, and LHS-3 > 50% lung involvement (32).

Immunohistochemistry of tissue sections. Lung tissues were obtained from 10 LAM patients enrolled in the protocol, and samples were acquired at the time of either lung transplantation (n = 8) or surgical lung biopsy (n = 2). We also analyzed tissue sections from 10 different patients with idiopathic pulmonary fibrosis. Further information is in the supplemental material and described in Ref. 32. Immunohistochemical assays were performed on formalin-fixed, paraffin-embedded tissue sections. Slides were processed for staining using established procedures. Antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), unless otherwise indicated. Dilutions of antibodies (vol/vol) were as follows: EPO (rabbit polyclonal H-162, 1:100), EPO receptor (EPOR; rabbit polyclonal, C-20; 1:100), and p-EPOR (Tyr 479) (rabbit polyclonal 1:100). For detection of bound antibodies, slides with tissue sections were washed four times with PBS and then incubated for 10 min at room temperature with biotinylated goat anti-rabbit antibody or anti-mouse antibody (horseradish peroxidase/diamino-benzidine detection system; Spring Bioscience, Fremont, CA). For negative controls, normal rabbit IgG was the primary antibody.

Laser-capture microdissection. LAM cells (5,000–7,000 spots) were collected using the VERITAS microdissection system (Arcturus Engineering, Mountain View, CA) with a laser power of 50–70 mW, duration of 2.5 ms, and laser spot size of 15.5 to 31.9 μm. Cells were pooled for isolation of RNA, using the Picopure RNA isolation kit (Arcturus Engineering), with incubation of cells in the extraction buffer at 42°C for 30 min. After centrifugation (800 g, 2 min), RNA was isolated from the supernatant, as described by the manufacturer. The entire process was completed within 2 h to minimize RNA degradation. Following quantification, RNA was stored at −80°C until analysis.

Cell sorting. Primary cells from lung tissues (cell lines LAM-1, -2, -3, and -4) were obtained as described previously (35). Cells were maintained on 100-mm plastic dishes and grown to near confluence in mesenchymal stem cell growth medium (MSCGM; Lonza Walkersville). To carry out cell sorting, after removal of medium, cells were washed with Dulbecco’s PBS (10 ml) without CaCl₂ and MgCl₂. Cells were detached from plates by incubation with 1 ml trypsin/EDTA at room temperature for 5 min; detachment was monitored by microscopic inspection. Trypsin action was terminated with addition of 10 ml of MSCGM. Cells were dispersed and centrifuged (500 g) to obtain a cell pellet that was resuspended in Dulbecco’s PBS without CaCl₂ and MgCl₂. For FACS analyses, cells (1 × 10⁶ cells in 500 μl) were reacted with anti-CD44 antibodies labeled with R-phycocerythrin and anti-CD44v6 antibodies labeled with fluorescein (clone F10–44–2 and VFF-7, respectively, from Invitrogen, Carlsbad, CA). Cell sorting was performed on a MoFlo flow cytometer (Dako-Cytomation, Fort Collins, CO), as described before (10).

RT-PCR. To detect mRNA for EPO or its receptor by RT-PCR, first-strand cDNA was primed with oligo(dT) and random primers using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). To increase the sensitivity of the RT-PCR assay and the precise calculation of the PCR products, we used fluorescently labeled primers in the reactions, and products were analyzed using the 3100 Genetic analyzer from Applied Biosystems. These plots show the peaks, representing the RT-PCR products, and the size standards. Amplification of GAPDH mRNA was routinely performed as a control, and incubations without reverse transcriptase or template were negative controls. Additional details are in the supplemental material.

Proliferation of cultured cells. Cells grown in MSCGM (Lonza Walkersville) were detached by trypsinization. Using the resulting cell suspension, 3,000 cells per 100 μl per well were placed in 96-well plates and allowed to attach for 16 h. The MSCGM was replaced with MSCGM without serum, and cells were allowed to grow for 16 h. Finally, cells were incubated with or without bromodeoxyuridine (BrDU) for 24 h in the presence of the indicated concentrations of EPO. To monitor DNA synthesis, BrDU incorporation was calculated by subtracting the absorbance of cells not incubated with BrDU from the ones incubated with BrDU, as previously reported.

Binding of EPO to collagen. We used collagen and Matrigel to assess EPO binding to extracellular matrix. To prepare the Matrigel matrix for EPO binding, 50 μl of rat Matrigel (3.4 mg/ml) were allowed to solidify on wells of 96-well plates. Binding of EPO to collagen type I was tested on 96-well plates. To coat the wells, 100 μl of rat collagen type I (BD Biosciences) in PBS (50 g/ml) were added to each well, followed by incubation for 2 h at room temperature. After removal of the solution, wells were washed twice with 300 μl of PBS.

Table 1. Red blood cell indexes, lung function, V̇O₂max, and serum erythropoietin levels in patients using or not using oxygen

<table>
<thead>
<tr>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
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<tbody>
<tr>
<td>Subjects, no.</td>
<td>180</td>
<td>33</td>
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<tr>
<td>Oxygen use</td>
<td>None</td>
<td>Intermittent</td>
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<tr>
<td>Age, yr</td>
<td>44.6 ± 9.5</td>
<td>45.9 ± 9.4</td>
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<tr>
<td>Hematocrit, %</td>
<td>40.7 ± 3.2</td>
<td>42.3 ± 3.2†</td>
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<tr>
<td>Hemoglobin, g/dl</td>
<td>13.6 ± 1.1</td>
<td>14.1 ± 1.1†</td>
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<tr>
<td>RBC, million/μl</td>
<td>4.56 ± 0.43</td>
<td>4.65 ± 0.32</td>
</tr>
<tr>
<td>PaO₂, Torr</td>
<td>83.5 ± 13.9</td>
<td>68.1 ± 12.1‡</td>
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<td>FEV₁, %predicted</td>
<td>79.5 ± 23.7</td>
<td>64.2 ± 23.4</td>
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<tr>
<td>DLCO, %predicted</td>
<td>78.2 ± 21.98</td>
<td>48.3 ± 13.9</td>
</tr>
<tr>
<td>V̇O₂max, %predicted</td>
<td>77.8 ± 20.0</td>
<td>59.9 ± 21.0‡</td>
</tr>
<tr>
<td>Erythropoietin, mU/ml</td>
<td>11.0 ± 5.5</td>
<td>10.9 ± 4.7</td>
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Values are means ± SD. RBC, red blood cell count; PaO₂, arterial blood oxygen tension; FEV₁, forced expiratory volume in first second; DLCO, diffusion capacity for carbon monoxide; V̇O₂max, peak oxygen uptake. †Significantly different from patients not using oxygen (P < 0.05). ‡Significantly different from the other two groups (P < 0.05).
containing Matrigel or collagen before quantification of EPO remaining in samples of medium. EPO was assayed by ELISA, as described by the manufacturer (R&D Systems). Data are from three experiments with triplicate assays.

Statistical methods. Patients were grouped in three categories based on levels of supplemental oxygen usage: none, only during physical activities and/or sleep, and essentially continuous. We compared hematocrits, hemoglobin levels, and RBC counts of the three groups. A trend test (Jonckheere-Terpstra) was used to evaluate correlation of RBC indexes with oxygen use. Multivariate regression analysis was employed to identify additional factors associated with each of the RBC indexes. Among the independent variables considered were

Fig. 1. A and B: relationship between red blood cell count and lung diffusion capacity (DLCO) for patients who did not use oxygen ($P = 0.002, n = 164; A$) and patients on oxygen therapy ($P = 0.004, n = 110; B$). C and D: relationship between red blood cell count and rate of decline of DLCO for patients who did not use oxygen ($P =$ nonsignificant, $n = 145; C$) and patients on oxygen therapy ($P = 0.0008, n = 99; D$).

Fig. 2. Activation of erythropoietin (EPO) receptor (EPOR) by EPO in lymphangioleiomyomatosis (LAM) cells. I. A: immunoreactivity of anti-EPOR antibodies with LAM cell nodular structures (black rectangle) and vascular cells (black arrow) of explanted LAM lung. B: immunoreactivity with anti-EPOR antibodies of endothelial cells (black arrow) and vascular smooth muscle cells. C: immunoreactivity with anti-EPOR antibodies of LAM cells (black square) and alveolar macrophages (arrowhead) of normal lung vasculature. II. A: immunoreactivity of spindle-shaped proliferative LAM cells (black rectangle) and type II pneumocytes (black arrow) of normal lung vasculature. Arrow shows the immunoreactivity of anti-EPOR antibodies with vascular endothelial cells. E and F: magnification $\times 40$. B and D: $\times 100$. II. A: immunoreactivity of alveolar space (blue circle) and vascular structures (black arrow) with anti-phospho (p)-EPOR (Tyr479) antibodies. B: immunoreactivity of spindle-shaped proliferative LAM cells (black rectangle) and type II pneumocytes (black arrowhead) with anti-p-EPOR (Tyr479) antibodies. C: staining of alveolar space (blue circle) or LAM cells (black rectangle). The immunohistochemical analysis, performed with samples from 10 patients, showed similar results. A–C: original magnification: $\times 40$. 

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body mass index, DLCO, FEV1, peak oxygen uptake, arterial oxygen tension (PaO2), oxygen saturation at rest and at peak exercise, and serum EPO levels. A logarithmic transformation was employed for skewed variables. A cluster analysis was used to group all available predictors into groups of similar (correlated) variables. The most statistically significant variable from each group was used in a multivariate regression analysis. All variables with \( P < 0.05 \) were retained in the model. Data are presented as means ± SD.

RESULTS

RBC indexes and severity of lung disease. To evaluate whether there was a relationship between RBC indexes and severity of lung disease, we analyzed pulmonary function and cardiopulmonary exercise data and RBC indexes from 277 LAM patients (mean age, 45.4 ± 10.0 yr; Table 1 and Supplemental Tables S1 and S2). The 277 patients were grouped, as shown in Table 1, based on use of oxygen therapy: 180 did not use oxygen (group I), 33 used oxygen intermittently (group II), and 64 used oxygen continuously (group III). Patients who used supplemental oxygen intermittently or continuously had higher hematocrit (\( P = 0.013 \) and \( P = 0.001 \), respectively) and hemoglobin (\( P = 0.014 \) and \( P = 0.001 \), respectively) levels than those who did not. Patients using supplemental oxygen continuously also had higher RBC counts (\( P = 0.027 \)) than patients who did not use oxygen.

RBC count was significantly correlated with DLCO for patients not on supplemental oxygen (\( P = 0.002 \), Fig. 1A), patients on supplemental oxygen (\( P = 0.004 \), Fig. 1B), and for both groups combined (\( P < 0.0001 \), Supplemental Fig. S1A).

Fig. 3. Detection of EPO and EPOR mRNA in LAM nodules and binding of EPO to collagen. A: detection of EPOR mRNA by RT-PCR in lung (normal), kidney (normal), pulmonary artery smooth muscle cells (PASM), A549, and laser-captured microdissected LAM cells. *EPOR peak. B: detection of EPO mRNA in total RNA obtained from human lung, human kidney, PASM, A549 cells, and laser-captured microdissected LAM cells. Markers are indicated with arrows and the number of base pairs: 160, 139, and 150. C: binding of human recombinant EPO to Matrigel and collagen at 24 and 37°C. The data represent three experiments run in triplicate samples. Values are means ± SE.

Fig. 4. Detection of EPO and EPOR mRNA in cultured LAM cells. A and B: cultured cells grown from LAM lungs were immunostained with CD44 and CD44v6 and separated by cell sorting (B). Detection of EPOR (C) and EPO (D) mRNA in the mixture of cells (control) or the indicated sorted cell populations are shown. The population of cells expressing EPO mRNA (CD44+, CD44v6+) differs from the CD44v6+ populations containing tuberous sclerosis complex 2 (TSC2) loss of heterozygosity (LOH) (CD44+, CD44v6−; CD44−, CD44v6+). Experiments were repeated four times with similar results using two different cell lines from two patients. *EPO or EPOR mRNA peak. 160-, 139-, and 150-bp markers are indicated in the chromatogram. SSC, side scatter; PE, phycoerythrin.
from each group. Percent-predicted DLCO was a significant predictor of survival or time to transplantation (32). Mental Fig. S1, a measure of severity of lung disease that is associated with the endothelial cells. This result contrasts with the finding that anti-EPO antibodies do not react with proliferating fibroblast of lung tissue sections from patients with idiopathic pulmonary fibrosis (Fig. 2I, E and F), a disease characterized by proliferation of fibroblast and large deposits of collagen (22). We observed immunoreactivity of anti-EPO with alveolar macrophages and type II pneumocytes of idiopathic pulmonary fibrosis histological sections (Fig. 2IF). We also observed immunoreactivity of LAM cells with anti-HIF-2α, but not with anti-HIF-1α antibodies (Supplemental Fig. S3).

We next investigated the activation of EPOR in LAM cells (Fig. 2I). EPOR is phosphorylated at multiple sites after binding of EPO (13). Phosphorylation of Tyr479 is responsible for EPOR activation and plays a role in cell proliferation (28, 52). While smooth muscle cells reacted weakly with anti-phospho-EPOR (Tyr479), the staining of alveolar cells was stronger (Fig. 2IA). Strong immunoreactivity with the antibodies against activated EPOR (Tyr479) was observed, however, in cells within the LAM lung nodules and in adjacent type II pneumocytes (Fig. 2IIIB). These data suggest that LAM cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>50 U/ml Proliferation, %</th>
<th>100 U/ml Proliferation, %</th>
<th>P Value</th>
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<tbody>
<tr>
<td>LAM-1*</td>
<td>115.0 ± 2.60</td>
<td>114.0 ± 17.50</td>
<td>0.54</td>
</tr>
<tr>
<td>LAM-2*</td>
<td>110.5 ± 9.70</td>
<td>138.4 ± 8.00</td>
<td>0.052</td>
</tr>
<tr>
<td>LAM-3*</td>
<td>114.8 ± 3.60</td>
<td>135.3 ± 4.00</td>
<td>0.014</td>
</tr>
<tr>
<td>LAM-4</td>
<td>128.8 ± 10.0</td>
<td>156.8 ± 6.20</td>
<td>0.032</td>
</tr>
<tr>
<td>PASM</td>
<td>129.10 ± 2.0</td>
<td>113.3 ± 0.80</td>
<td>0.008</td>
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</table>

Values are triplicate measurements ± SE. Cells obtained from four different patients were stimulated with different concentrations of erythropoietin (0.1, 1.0, 2.5, 5.0, 10.0, 50.0, and 100.0 U/ml). Concentrations below 50 U/ml were without effect. *The cell lines used for erythropoietin receptor activation and FACS cell sorting. LAM, lymphangioleiomyomatosis; PASM, pulmonary artery smooth muscle cells. P values represent significant increases in proliferation compared with vehicle control.

Lower resting PaO2 while breathing room air was also associated with higher RBC count (P = 0.0002), hemoglobin (P < 0.0001) (data not shown), and hemoglobin (P < 0.001) (data not shown). The resting PaO2 was a marker of lung disease severity (Supplemental Fig. S1C) and was significantly negatively correlated (P = 0.007, n = 50) with the LHS (Supplemental Fig. S1D), a measure of severity of lung disease that is a predictor of survival or time to transplantation (32).

To further examine the relationship between RBC parameters and measures of disease severity, we used cluster analysis to establish nine sets of correlated variables. A multivariate analysis was performed using the most significant variable from each group. Percent-predicted DLCO was a significant predictor of the hematocrit (P < 0.0001), hemoglobin (P < 0.0001), and RBC count (P < 0.0001). Oxygen saturation at peak exercise was also significantly correlated with RBC count (P < 0.0001). To assess the relationship between RBC count and rate of progression of lung disease, we correlated RBC count with the yearly rate of DLCO decline. While this relationship did not reach statistical significance for patients not receiving supplemental oxygen (Fig. 1C), the correlation between RBC count and the yearly rate of decline in DLCO was significant for patients who used supplemental oxygen (P = 0.0008, Fig. 1D) and for all patients combined (P < 0.003, Supplemental Fig. S1B).

Our data show that higher RBC indexes were associated with a greater severity of lung disease and a greater rate of decline in lung function. Since hypoxia increases RBC indexes, through increased synthesis of EPO (13, 38), the accelerated loss of lung function in those with erythrocytosis could be due to a growth-enhancing effect of EPO on LAM cells. The next step of our study was, therefore, to determine if EPO effects could be found in LAM lung nodules and on LAM cells in culture.

Expression of EPOR, EPO, and components of an activated EPO-dependent signaling pathway by LAM cells. Positive immunoreactivity for the EPOR was observed in both epithelioid and spindle-shaped LAM cells in LAM lung nodules (Fig. 2IA) and, to a lesser extent, in normal vascular smooth muscle and endothelial cells, which can be appreciated in Fig. 2IB. The immunostaining of these cell types is in agreement with previous reports (2). Cultured cells grown from explanted LAM lungs reacted with the anti-EPOR antibody (~90% immunoreactive cells), as did pulmonary artery smooth muscle cells (PASM) (data not shown). The weak immunoreactivity of smooth muscle cells to anti-EPOR appears to contrast with the expression of mRNA for EPOR present in PASM; however, this discrepancy could be due to the sensitivity of the antibody and/or type of molecular analysis (below). This result is consistent with expression of EPOR in different tumor cell lines (34).

Immunohistochemical studies of sections of explanted LAM lungs showed reactivity with antibodies against EPO in LAM lung nodules (Fig. 2IC); type II pneumocytes were not significantly stained (Fig. 2IC). To identify the staining of normal lung vasculature, we focused on anti-EPO immunoreactivity of smooth muscle and endothelial cells that were adjacent to LAM nodules (Fig. 2ID), and we found that the staining was associated with the endothelial cells. This result contrasts with the finding that anti-EPO antibodies do not react with proliferating fibroblast of lung tissue sections from patients with idiopathic pulmonary fibrosis (Fig. 2I, E and F), a disease characterized by proliferation of fibroblast and large deposits of collagen (22). We observed immunoreactivity of anti-EPO with alveolar macrophages and type II pneumocytes of idiopathic pulmonary fibrosis histological sections (Fig. 2IF). We also observed immunoreactivity of LAM cells with anti-HIF-2α, but not with anti-HIF-1α antibodies (Supplemental Fig. S3).

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![Fig. 5. Activation of EPOR in cells cultured from LAM lungs. Cells cultured from LAM lungs were grown as described in MATERIALS AND METHODS. Cells (5 × 10⁵) were allowed to adhere to 100-mm plastic dishes for 24 h in complete media (CM). Then cell culture media was replaced with basal media without serum and incubated for 18 h. Cells were incubated without (vehicle, V) or with 100 U/ml of EPO for the indicated times (0, 10, 20 min) and in CM (20 min). Cells were lysed in mammalian protein extraction reagent (M-PER) containing protease inhibitors and phosphatase inhibitors. The experiment was repeated twice with different cell lines. IB, immunoblot.](http://ajplung.physiology.org/)
have EPOR and the activated pathway necessary for EPO-induced cell proliferation (21).

We observed that, while ~20% of the cells were double stained for EPOR and proliferating cell nuclear antigen (PCNA), <10% showed immunostaining of activated EPOR and PCNA (Supplemental Fig. S2f), suggesting that other factors are important to the proliferation of LAM cells. However, we observed activation of STAT-5 correlating with an EPO effect (Supplemental Fig. S2i).

EPOR mRNA was also detected in total RNA from LAM cells collected by laser-capture microdissection from LAM nodules (Fig. 3A, bottom graph), and in total RNA extracted from lung, kidney, PASM, and A549 cells (human lung epithelial adenocarcinoma cells). By contrast, EPO was not produced by the same microdissected LAM cells (Fig. 3B). These data led us to propose that EPO could be associated with the abundant collagen present in LAM lesions. Thus we tested the ability of recombinant human EPO to bind collagen and Matrigel. EPO was bound to collagen and to Matrigel, suggesting that EPO may be associated with the extracellular matrix (Fig. 3C).

Our laboratory has shown that cells from LAM lung explants that react with antibodies against the membrane-associated CD44v6 molecule have dysfunctional TSC2 (35). Therefore, we assessed the presence of EPO and EPOR in cells sorted by FACS using CD44 and CD44v6 antibodies. Cells were gated to separate four cell populations (CD44+/CD44v6+, CD44−/CD44v6−, CD44−/CD44v6+, and CD44+/CD44v6−) from the heterogeneous LAM cell culture (Fig. 4, A and B). RNA isolated from each of these populations was shown by RT-PCR to contain EPOR mRNA (Fig. 4C). Cultured LAM cells (CD44+/CD44v6+ and CD44−/CD44v6−), as well as laser-captured microdissected LAM cells, did not produce EPO (Fig. 4D). However, the CD44+/CD44v6− cells produced EPO (Fig. 4D). Thus, whereas LAM cells possess EPOR, they did not produce EPO. They could be affected by the production of EPO by non-LAM cells in the lung.

EPO increases proliferation of TSC2−/− cells. Cells grown from LAM lung explants, which exhibit loss of heterozygosity for TSC2, were used to test the effects of EPO on proliferation in vitro. We carried out these experiments under normoxic conditions to avoid the effect of endogenously produced EPO and hypoxia-induced EPOR. Table 2 shows that EPO increased proliferation of cells grown from explanted LAM lungs similar to the results obtained with TSC2−/− skin fibroblasts (see Fig. 7). EPO caused proliferation of cells grown from explanted LAM lungs similar to the results obtained with TSC2−/− skin fibroblasts because they are a more homogeneous human cell population with a dysfunctional TSC2-mTOR pathway, as determined by the lack of tuberin by Western blotting and increased phosphorylation of S6 compared with their tuberin-containing control fibroblast from patient normal-appearing skin (see Fig. 7).

Fig. 6. Cultured LAM cells reactive to anti-CD44v6 and anti-EPOR antibodies showed TSC2 LOH. A and D: cells sorted by SSC and forward scatter (FSC). B and E: the different cell populations selected by R1. B: the number of cells used for DNA analysis: CD44v6+/EPOR− (29,304); CD44v6+/EPOR+ (7,304); CD44v6+/EPOR− (2.3 × 106); CD44v6−/EPOR− (749). E: the number of cells: CD44v6+/EPOR− (2,384); CD44v6+/EPOR+ (4,014); CD44v6−/EPOR− (0.9 × 106); CD44v6−/EPOR+ (338). C and F: the microsatellite instability in chromosome 16 (D16S3395). The chromosomal regions were amplified using the primer pair D16S3395 forward (5′-CTA ACC CTC AGA AGT GTT CTG-3′) and D16S3395 reverse (5′-Fam-CCT GCC AGT AAG TCC TGA AA-3′). The arrowhead shows the diminished allele. \(Q_{LOH}\) was determined as the ratio of fluorescence intensities of each allele: LAM or sorted cells (L) cells to that in whole blood (N) from the same patient \([L_1/L_2]/(N_1/N_2)\). L1 is the diminished allele. Values of ≥0.6 were scored as LOH. The data reflect findings in two patients. A third patient gave similar results. AU, arbitrary units.
Although these EPO concentrations used are higher than the ones observed in serum, as noted in this report and elsewhere, EPO appears to be concentrated in certain tissues, perhaps by binding to extracellular matrix (20). It is noteworthy that high concentrations of EPO have been used by many groups to determine the effect of EPO on nonhematological cells (46).

Furthermore, a time-dependent activation of EPOR in two LAM lung cell lines showed the functionality of EPO on cultured cells (Fig. 5). Interestingly, we noted activation of EPOR even in the absence of EPO, suggesting a constitutive activation of the receptor in the same way as breast cancer cells (16). Although we observed activation of EPOR by EPO, these heterogeneous populations of cells containing a minor percentage of LAM cells would not be suitable to investigate cell signaling processes in a quantitative manner.

Because these cells cultures contain LAM and non-LAM cells, we FACSc-sort cells based on their immunoreactivity to anti-EPOR and anti-CD44v6. Figure 6 shows that CD44v6+/EPOR+ and CD44v6−/EPOR+ cells possess TSC2 loss of heterozygosity, suggesting that LAM cells contain EPOR.

To verify that EPO affected the rate of proliferation of TSC2−/− cells, we used homogeneous TSC2−/− cells derived from a TSC skin tumor (periungual fibroma). These cells lack tuberin due to inactivating mutations in both TSC2 alleles (Fig. 7B), as has been reported in lung LAM cells by Carsillo et al. (8) and have phosphorylated pS6 due to activation of the mTOR pathway. We showed that TSC2+/+ and TSC2−/− cells produce EPOR (Fig. 7A, left) similar to HepG2; however, HepG2, but not TSC2−/− cells synthesize measurable amounts of EPO under similar conditions (Fig. 7A, middle). The lack of EPO expression in skin fibroblasts from TSC patients is consistent with the lack of staining for EPO in normal skin fibroblasts (23). Furthermore, comparison of gene expression between the TSC2+/+ and TSC2−/− cells showed that the EPOR is approximately threefold higher in TSC2−/− cells than in TSC2+/+ (data not shown).

In the presence of EPO, the TSC2−/− skin tumor cells had a higher proliferation rate, with a two- to threefold increase, than did fibroblasts grown from normal-appearing skin (the experiments were repeated four times in triplicate assays).

DISCUSSION

This study demonstrates the presence of activated EPOR in LAM lung lesions and in LAM cells in culture, along with the existence, by immunohistochemistry, of an activated EPO signaling pathway, which could lead to LAM cell proliferation and the progressive growth of LAM lesions. Furthermore, we show that EPO promoted the growth of LAM lung cells and TSC2−/− skin tumor cells. Although we found no evidence for the production of EPO by LAM cells, our data suggest that EPO production either by the renal cortex or non-LAM cells in the lung could enhance LAM cell proliferation and thereby accelerate progression of the disease. Elevated red cell count, probably in response to hypoxia, was associated with more rapid decline in lung function, establishing a link between the pathophysiology of LAM and activation of signaling pathways that promote LAM cell growth.

This finding is unique because it establishes a correlation between hypoxia-enhanced erythrocytosis and accelerated loss of lung function. That is, erythrocytosis, which is presumably caused by EPO, appears to be associated with lung function decline. Indeed, as previously reported (48), we had expected patients with lower DLCO to have a slower rate of decline in DLCO. Instead, we found that patients with higher RBC indexes, despite having lower DLCO, had faster rates of DLCO decline.

Although the RBC counts were within a normal range, the ability to achieve statistically meaningful data reflected the fact that our relatively large cohort of LAM patients was willing to return to the NIH for multiple visits, enabling us to follow disease progression and monitor biomarkers.

In other lung diseases, the rate of decline in function did not increase as the disease progressed. For example, in 43 patients with chronic obstructive pulmonary disease due to α1-antitrypsin deficiency, the decline in FEV1 occurred in subjects with the least initial impairment, i.e., greater FEV1 (11). Similarly, in a group of 543 subjects participating in a longitudinal...
epidemiological study, the rate of decline in $D_{LCO}$ in smoking subjects with lower $D_{LCO}$ was not different from that of nonsmoking subjects with higher $D_{LCO}$ (41). More importantly, in LAM, we found that the rate of $D_{LCO}$ decline was greater in patients with less severe disease, i.e., higher initial values of $D_{LCO}$ and FEV₁ (47). EPO levels are not consistently elevated in pathological processes, and the relationship among red cell indexes, hypoxia, and EPO levels are highly variable (9, 17, 44). The fact that we did not observe high levels of EPO in these patients could be correlated to the short half-life of EPO in the blood circulation, which is 1.51–2.92 h (12). The elevated RBC indexes support the hypothesis that a functionally relevant EPO elevation had occurred and was driving red cell synthesis. This has been reported in patients with chronic obstructive pulmonary disease, in whom the relationship between EPO levels, degree of hypoxemia, and red cell mass is highly variable (17, 44, 50). The lack of correlation between daytime EPO levels and erythrocytic responses (6, 14) could also be due to the intermittent nature of hypoxemia and the circadian rhythm of EPO secretion (26). High red blood indexes were more often found in patients with hypoxemia and were associated with a greater rate of decline in lung function.

The physiological response to hypoxia includes activation of EPO gene transcription by HIF-1α, leading to an increase in EPO production by the renal cortex, promoting growth of erythropoietic progenitors by suppressing their apoptosis (26, 51). Erythrocytosis as a consequence of increased EPO levels is a mechanism of adaptation to hypoxia (18), occurring both in healthy subjects exposed to high altitude and in patients with cardiopulmonary diseases and decreased arterial oxygen content.

Although the kidney is the main source of circulating EPO (5), EPO can be produced in other organs under the regulation of HIFs. We found that HIF-2α is present in LAM lung nodules, suggesting that this transcription factor plays a role in the hypoxic response; however, we were unable to detect EPO mRNA in LAM cells, suggesting that other factors are required for EPO expression. In fact, a similar discrepancy between the presence of HIF-2α and lack of EPO production has been observed in kidney capillary endothelial cells (7). Thus we propose a pulmonary and an extrapulmonary source of EPO. The presence of the activated EPOR phosphorylated at Tyr479 within LAM nodules is sufficient to cause an increase of cell proliferation (7, 28). The fact that we detected EPOR on LAM cells in the presence of low levels of EPO suggests that EPOR could be activated by 1) low concentrations of EPO; 2) other ligands; 3) the increased amounts of EPOR; 4) receptor cross-activation; and 5) activated receptor maintained after dissociation or degradation of ligand. The mechanism for EPOR activation on LAM cells remains to be determined.

Although we find a small number of cells immunoreactive to antibodies directed to activated EPOR and proliferative cells (PCNA-positive cells), it is possible that proliferative LAM cells could be driven by multiple factors, such as angiotsenin II (49), or by the alterations of the TSC complex (19, 37). We found no evidence, however, for the production of EPO mRNA by LAM cells and conclude that EPO is synthesized elsewhere by non-LAM lung cells, such as kidney, liver, smooth muscle, and vascular endothelial cells, which may exert a paracrine effect on LAM cells (13). It is possible that EPO binds to the extracellular matrix, as shown by binding of human recombinant EPO to collagen gels (Fig. 3). Similarly, it was previously found that basic fibroblast growth factor bound by extracellular matrix was protected from degradation (39). Thus EPO could bind to the large deposits of collagen present in LAM lungs (22). This could increase the likelihood of chronic stimulation of the LAM cells, independent of fluctuations of EPO concentration in the circulation, secondary to episodic hypoxia. In the present study, we found that EPO enhanced the proliferation rate of cells grown from LAM lungs cells and human skin TSC2−/− cells. EPO may also affect cell migration (31).

We conclude that a hypoxia-mediated increased production of EPO by the kidney cortex or by non-LAM cells within the lungs may promote LAM cell proliferation and thereby increase the rate of disease progression. In addition, putative hypoxic regions of LAM nodules may offer a selective advantage for abnormal cells to grow, as seen in tumors. Administration of oxygen, by suppressing EPO production, could potentially slow the growth and proliferation of LAM cells with beneficial effects on disease progression.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Erythropoietin and Lymphangioleiomyomatosis


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