Proproliferative phenotype of pulmonary microvascular endothelial cells

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Solodushko V, Fouty B. Proproliferative phenotype of pulmonary microvascular endothelial cells. Am J Physiol Lung Cell Mol Physiol 292: L671–L677, 2007.—Endothelial cells perform a number of important functions including release of vasodilators, control of the coagulation cascade, and restriction of solutes and fluid from the extravascular space. Regulation of fluid balance is of particular importance in the microcirculation of the lung where the loss of endothelial barrier function can lead to alveolar flooding and life-threatening hypoxemia. Significant heterogeneity exists between endothelial cells lining the microcirculation and cells from larger pulmonary arteries, however, and these differences may be relevant in restoring barrier function following vascular injury. Using well-defined populations of rat endothelial cells harvested from the pulmonary microcirculation [pulmonary microvascular endothelial cells (PMVEC)] and from larger pulmonary arteries [pulmonary artery endothelial cells (PAEC)], we compared their growth characteristics in low serum conditions. Withdrawal of serum inhibited proliferation and induced G0/G1 arrest in PAEC, whereas PMVEC did not undergo G0/G1 arrest and continued to proliferate. Consistent with this observation, PMVEC had an increased cdk4 and cdk2 kinase activity with hyperphosphorylated [inactive] retinoblastoma (Rb) relative to PAEC as well as a threefold increase in cyclin D1 protein levels; overexpression of the cdk inhibitors p21Cip1/Waf1 and p27Kip1 induced G0/G1 arrest. While serum withdrawal failed to induce G0/G1 arrest in nonconfluent PMVEC, confluence was associated with hyperphosphorylated Rb and growth arrest; loss of confluence led to resumption of growth. These data suggest that nonconfluent PMVEC continue to proliferate independently of growth factors. This proliferative characteristic may be important in restoring confluence (and barrier function) in the pulmonary microcirculation following endothelial injury.

The pulmonary circulation is a high-compliance, low-resistance vascular bed designed to facilitate gas exchange. Endothelial cells line the pulmonary vasculature and perform a number of functions to effectively match perfusion with ventilation. Release of vasodilators such as prostacyclin and nitric oxide, control of the coagulation cascade to prevent in situ thrombosis, and restriction of solutes and fluids to the vascular space and out of the interstitium and alveoli are among these important functions. The development of a relatively impermeable endothelial layer is particularly important in the pulmonary microcirculation: the arterioles, capillaries, and venules that participate in gas exchange. Disruption of the endothelial lining in this region in response to sepsis, trauma, pancreatitis, or other insults can lead to widespread alveolar flooding and the development of life-threatening hypoxemia (27). Any disruption of endothelial confluence in the microcirculation must be rapidly repaired to restore proper gas exchange.

A large body of work suggests that there are important differences in how vascular endothelial cells within different regions of the lung function. Endothelial cells obtained from extra-alveolar pulmonary arteries (pulmonary artery endothelial cells or PAEC) can be distinguished from endothelial cells residing in pulmonary vessels less than 25 μm (pulmonary microvascular endothelial cells or PMVEC) by both in vitro and in vivo lectin binding (15) and responses to agonists such as thrombin (6), atrial natriuretic protein (29), and thapsigargin (5, 7, 28). In addition, endothelial cells in the pulmonary microcirculation are an integral part of the barrier that prevents fluid translocation into the interstitium and alveoli; when studied in situ (22) and in vitro (14), PMVEC develop a significantly tighter, less permeable barrier than PAEC. Since endothelial cells in the pulmonary microcirculation perform a critical role in excluding fluid from the gas-exchange region, any injury that disrupts confluence can be devastating. As a result, the reparative mechanisms of endothelial cells in this critical region might be expected to be distinct from endothelial cells in less critical areas such as the larger pulmonary vessels. Such reparative mechanisms would include the ability to proliferate rapidly following injury to restore endothelial confluence even if growth conditions are not optimal due to disruption of blood flow to affected vessels.

We examined the growth characteristics of PMVEC in a mitogen-poor environment and compared them to cells obtained from larger pulmonary arteries. We demonstrated that nonconfluent PMVEC proliferated significantly faster than PAEC in low-serum conditions, and, unlike PAEC, they did not undergo G0/G1 arrest following serum withdrawal. This unusual ability to proliferate in low serum appeared to be due in part to an increase in G1 cyclin-cyclin-dependent kinase (cdk) complex activity that led to the hyperphosphorylation and inactivation of the tumor suppressor retinoblastoma (Rb). A marked increase in cyclin D1 protein expression also distinguished PMVEC from PAEC. Once PMVEC reached confluence, however, they did undergo G0/G1 arrest demonstrating that control of cell proliferation is maintained in these cells. These results suggest that PMVEC are designed to grow rapidly following the disruption of confluence, a characteristic that would be critical in restoring normal gas-exchange to the lung following endothelial injury.

MATERIALS AND METHODS

DMEM, Ipegal, propidium iodide, RNase, and 5'-bromo-2'-deoxyuridine (BrdU) were all from Sigma (St. Louis, MO). Trypsin—
EDTA and 1-glutamine were from Gibco (Grand Island, NY). FBS was from Gemini (Woodland, CA), and polyvinylidene difluoride (PVDF) membrane was from Amersham (Buckinghamshire, England). SuperSignal West Dura and SuperSignal West Femto were both from Pierce (Rockford, IL). Antibodies used included p27<sup>kip</sup> from Abcam (1-C-19), p21<sup>cip1</sup>/waf1 from Santa Cruz Biotechnology (Santa Cruz, CA), p27<sup>kip1</sup> from Cell Signaling Technology (Danvers, MA), and anti-β-actin from Sigma. PMVEC were isolated and cultured using a modified method described by King et al. (15). The endothelial cell phenotype was confirmed using SE, uptake of 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-conjugated antibodies used were donkey α-mouse and donkey α-rabbit from Jackson Labs (West Grove, PA).

**Cell culture.** PAEC were obtained from main pulmonary arteries as previously described (15). The endothelial cell phenotype was confirmed by acetylated LDL uptake, factor VIII-Rag immunocytochemical staining, and the absence of immunostaining with smooth muscle cell α-actin antibodies. PMVEC were isolated and cultured using a modified method described by King et al. (15). Cultures were characterized using SE, uptake of 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled LDL, and a lectin-binding panel and were routinely passaged. PMVEC stained positive for the lectin Glycine max and Griffonia simplicifolia, whereas PAEC stained positive for Helix pomatia. Both PMVEC and PAEC stained positive for endothelial nitric oxide synthase, platelet/endothelial cell adhesion molecule-1, and vascular endothelial-cadherin.

**Cell proliferation.** All cell lines were grown in humidified incubators at 37°C in 5% CO<sub>2</sub>. PAEC and PMVEC were plated at various densities, serum-starved for 72 h in DMEM with 0.1% FBS, and then exposed to experimental conditions. Cells were harvested by 0.05% trypsin/0.53 mM EDTA digestion and counted with Coulter Z1 (Coulter Electronics). Counts were made in triplicate.

**Adenoviral transfection.** Rat PAEC or PMVEC were infected for 4 h at a multiplicity of infection (MOI) of 100 with a replication-defective adenovirus serotype 5 containing a human p27<sup>kip1</sup>, p21<sup>kip1</sup>/waf1, or placental alkaline phosphatase (used as a control) cDNA driven by a cytomegalovirus promoter (obtained from the University of Michigan Vector Core, Ann Arbor, MI) (25). Cells were then washed and serum-starved in DMEM/0.1% FBS for 72 h before being studied.

**Western blotting.** Cells were harvested and lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin), incubated on ice for 10 min, and centrifuged at 13,000 g to clear the lysates. Protein content from total cell lysates was determined by Bradford assay, and 30–50 μg were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated in blocking solution and probed with primary antibodies overnight. Positive antibody reactions were visualized using peroxidase-conjugated secondary antibodies and a SuperSignal chemiluminescence detection system (Pierce) according to the manufacturer’s instructions.

**Flow cytometry.** Cells were digested with trypsin-EDTA from culture plates, and the trypsin was inactivated by addition of 10% FBS, washed with PBS by low centrifugation, and incubated in Krishan’s solution [50 μM L-methylphenylisopropyl (IP) 0.1% sodium citrate, 20 μg/ml RNase A, 0.3% Fetal Blocking Solution overnight at 4°C (16). Cells were analyzed directly by FACScan in the University of South Alabama Flow Cytometry Core.

**BrdU incorporation.** For BrdU and PI labeling, cells were pulse treated for 10 min with 1 mM BrdU/Brdu, washed three times with PBS, and harvested as described above. Cells were slowly added to 5 ml of 70% ethanol (~20°C) while maintaining a vortex. After 30 min of incubation (fixation), cells were collected by centrifugation (500 g, 10 min, 4°C). One milliliter of 2N HCl/Triton X-100 was slowly added to the cells. After a 30-min incubation at room temperature (to produce single-stranded DNA), cells were collected and resuspended in 1 ml of 0.1M Na<sub>2</sub>SO<sub>4</sub> × 10H<sub>2</sub>O, pH 8.5, to neutralize acid and were collected again. Cells were incubated with an anti-BrdU antibody for direct immunofluorescence staining. Cellular DNA was stained with 10 mM/l PI for 20 min. The samples were analyzed by fluorescence-activated cell sorting using FACScan (Univ. of South Alabama Flow Cytometry Core) for two-parameter dot plot histogram analysis (BrdU incorporation vs. DNA content).**

**Immunoprecipitation and kinase activity assay.** Cell lysate (200 μg) was incubated with 2 μg of cdk2 or cdk4 antibody (rabbit polyclonal) and Protein A-Sepharose beads (Santa Cruz) overnight at 4°C. Beads were washed twice with RIPA buffer and twice in cold kinase assay buffer [50 mM/l HEPES (pH 7.5), 10 mM/l MgCl<sub>2</sub>, 2.5 mM/l EDTA, 1 mM/l DTT, 10 mM/l β-glycerophosphate, 1 mM/l NaF, 0.1 mM/l sodium orthovanadate, and 20 μg/ml ATP]. Samples were resuspended in 30 μl of kinase assay buffer containing 5 μg of histone H1 (Upstate, Lake Placid, NY) for cdk2 activity or 2 μg of glutathione S-transferase (GST-Rb) (Santa Cruz) for cdk4 activity and 10 μg of [γ<sup>32</sup>P]ATP (Amersham Pharmacia Biotech); after incubation at 30°C for 30 min with occasional mixing, reactions were stopped by adding 30 μl of hot 2× Laemmli sample buffer and boiling for 5 min. Samples were resolved by SDS-PAGE, and phosphorylated proteins were detected by autoradiography.

**Statistical methods.** Data are expressed as means ± SE. Cell growth and changes in cell cycle profile were compared using ANOVA combined with Fisher post hoc analysis, with a P value < 0.05 considered significant. BrdU incorporation, protein expression levels, and in vitro kinase activity were compared using a two-tailed unpaired t-test with a P value < 0.05 considered significant.

### RESULTS

**Increased proliferation of PMVEC in low serum.** Cells were plated at low confluence (5–10%) and exposed to 0.1% serum. Figure 1A demonstrates that cells obtained from the microcirculation (PMVEC) had an increased growth rate compared with those obtained from the larger pulmonary artery (PAEC) when grown in 0.1% serum. To minimize the well-to-well variation of cell counts, we stably transfected PMVEC and PAEC with a retrovirus expressing green fluorescent protein (GFP), confirmed that changes in GFP intensity correlated with changes in cell number (data not shown), and then used the intensity of GFP to confirm the increased proliferation of PMVEC relative to PAEC (Fig. 1B). Consistent with this finding of increased proliferation, 37% of PMVEC were still in S phase 3 days after serum withdrawal (Fig. 2, A and B). In contrast, PAEC had only 11% of cells in S phase in low-serum conditions. These figures also demonstrate a significantly higher number of PAEC in G<sub>0</sub>/G<sub>1</sub> (86.3% ± 6%) relative to PMVEC (42.2% ± 8%).

**PMVEC progress through S phase in low serum.** One possible explanation for the increased number of cells in S phase in PMVEC grown in 0.1% serum was the development of an S phase arrest. To assess progression through S phase, PMVEC and PAEC were placed in low serum for 3 days, pulsed with BrdU for 10 min, fixed, incubated with an anti-BrdU antibody, and BrdU incorporation assessed by flow cytometry. Figure 2, C and D, demonstrate that even in 0.1% serum, PMVEC were still incorporating BrdU, demonstrating that these cells were progressing through S phase. Some BrdU incorporation also occurred in PAEC but to a much lesser degree. Combined with the cell proliferation data, this experiment supports the conclusion that PMVEC continued to grow even in low serum.
Increased kinase activity in PMVEC. The inactivation (hyperphosphorylation) of Rb occurs through the sequential action of two G1 protein complexes, cyclin D-cdk4 and cyclin E-cdk2 (23, 24). In the absence of mitogens, these cyclin-cdk complexes are inactive in most cells, and, as a result, Rb is hypophosphorylated leading to G0/G1 arrest. In an attempt to explain the hyperphosphorylation of Rb in PMVEC despite the lack of mitogens, we examined the cdk2 and cdk4 in vitro kinase activity of PAEC and PMVEC after 3 days of low serum. Cdk2 kinase activity was approximately fourfold and cdk4 kinase activity was approximately twofold higher in PMVEC than in PAEC (Figs. 3C and 4, C and D).

Overexpression of cdk inhibitors blocked hyperphosphorylation of Rb and induced G0/G1 arrest. To determine whether increased activity of these G1 kinases was the reason for the hyperphosphorylation of Rb in low-serum conditions, we examined the effect of overexpressing the cdk inhibitors p21cip1/waf1 or p27Kip1 on Rb phosphorylation and cell cycle profile. p21cip1/waf1 and p27Kip1 are members of the Kip/Cip family that can bind both cyclin D-cdk4 and cyclin E-cdk2 complexes (23, 24). Using a replication-deficient adenovirus encoding the human cDNA for p21cip1/waf1 or p27Kip1 (obtained from the Univ. of Michigan Vector Core), we infected PMVEC at a MOI of 100. Overexpression of the cdk inhibitors reduced the hyperphosphorylation of Rb in the PMVEC (Fig. 4A) and increased the percent of cells in G0/G1 (Fig. 4B) relative to the control adenovirus that encoded the gene for human placental alkaline phosphatase. Whereas cdk4 kinase activity was not significantly reduced (Fig. 4C), cdk2 kinase activity was almost completely inhibited by the cdk inhibitors (Fig. 4D). [This preferential inhibitory effect of the Cip/Kip cdk inhibitors on cdk2 (relative to cdk4) activity, despite similar levels of cdk inhibitor-complex binding, has been reported by others (2, 12) and likely relates to differences in whether the cdk active site is blocked by the inhibitor.] These experiments demonstrate that in low serum, PMVEC have increased cdk4 and cdk2 kinase activity that leads to the inactivation of Rb. By reducing the kinase activity of these cyclin-cdk complexes (principally cdk2), Rb remained in its hypophosphorylated state, and PMVEC became arrested in G0/G1.

Confluence arrests PMVEC in G0/G1. The previous experiments suggested that PMVEC, unlike PAEC, do not growth arrest when placed in low serum. We then tested whether confluence, which like serum withdrawal, is a strong inducer of cell cycle arrest in many cells (4), could induce growth arrest in PMVEC. Cells were plated sparsely and allowed to grow to a confluent monolayer. Cells were harvested at both 20% and 100% confluence, stained with PI, and analyzed by flow cytometry. In contrast to the (lack of) effect of serum withdrawal, the development of a cell monolayer caused a marked G0/G1 cell cycle arrest in PMVEC (Fig. 5, A and B). Consistent with this finding, Rb became hyperphosphorylated at confluence (Fig. 5, C and D). Establishing a monolayer had no effect on cyclin D1 protein expression but caused a significant up-regulation of the cdk inhibitor p27Kip1, something not seen with serum withdrawal (Fig. 5C). When confluent (arrested) cells were then trypsinized and replated at low density in 0.1% serum, the number of dividing cells increased within 30 h (Fig. 6, A and B).
DISCUSSION

Unique among the vascular beds, loss of endothelial integrity in the pulmonary microcirculation can lead to rapidly fatal hypoxemia. The ability to regain confluence and reestablish barrier function is central to restoring proper gas exchange. Such repair would require cells to undergo rapid proliferation to reestablish confluence and might frequently occur in suboptimal growth conditions, particularly if blood flow is reduced to injured vessels due to vascular obstruction or vasoconstriction.

Fig. 2. Serum withdrawal does not induce G0/G1 cell cycle arrest in PMVEC. Cells were plated in 0.1% serum for 3 days, harvested with trypsin, stained with propidium iodide, and analyzed by flow cytometry. A: representative cell cycle profiles. B: statistical aggregate of 4 distinct experiments. There was a statistically significant increase in PMVEC in S and G2/M and statistically fewer PMVEC in G0/G1 compared with PAEC. After 3 days in 0.1% serum, cells were pulsed with 1 μmol 5’-bromo-2’-deoxyuridine (BrdU) for 10 min, harvested, stained with an anti-BrdU antibody and propidium iodide, and analyzed by flow cytometry. C: representative flow cytometry data with intensity of BrdU staining on the y-axis and propidium iodide on the x-axis. D: statistical data from 6 distinct experiments (*P < 0.05).

Fig. 3. Hyperphosphorylated (inactive) retinoblastoma (Rb) with increased cdk4 and cdk2 kinase activity in PMVEC in 0.1% FBS. Cells were plated and placed in 0.1% serum for 3 days, and cell lysates were harvested. A: Western blot (representative blot from 4 different experiments). B: densitometry of Western blots (n = 4 with *P < 0.05). C: representative blot from in vitro assay analyzing cdk4 and cdk2 kinase activity in PMVEC compared with PAEC in 0.1% serum (n = 4). IP, immunoprecipitation; Rb-P, phosphorylated retinoblastoma; MV, microvascular cells; PA, pulmonary artery cells; CyD1, cyclin D1; His H1, histone H1.
Mechanisms that allow cells to undergo rapid proliferation would come at a metabolic cost, however, and might not be present in all endothelial cells, but would be reserved for critical locations. In this paper, we demonstrate that endothelial cells derived from the pulmonary microcirculation proliferate in a nonconfluent state even when there is minimal mitogenic stimulation. The presence of increased cyclin D1 levels along with the failure of p27Kip1 to upregulate in low-serum conditions in PMVEC appears to lead to the presence of active G1 cyclin-cdk complexes. These complexes inactivate the tumor suppressor, Rb, which leads to the proliferative phenotype that distinguished endothelial cells in the pulmonary microcirculation from those in larger pulmonary arteries.

In vivo, an endothelial cell in the pulmonary microcirculation will see a higher PO2 and a lower PCO2 than endothelial cells from the conduit pulmonary arteries, however. Therefore, we considered whether the proliferative advantage of the PMVEC relative to the PAEC in our experiments might relate to the oxygen/carbon dioxide environment of our cell culture conditions (21% oxygen, 5% CO2, balance nitrogen) rather than to intrinsic differences in proliferation. Therefore, in a single experiment, we assessed the growth of...
PMVEC and PAEC in gas conditions more consistent with that seen in vivo by the PAEC (P_{O2}: 40 mmHg/P_{CO2}: 47 mmHg, balance nitrogen). Similar to our results in room air, PMVEC maintained the approximately fourfold proliferative advantage at 5 days. This suggests that the phenotypic difference between PMVEC and PAEC is intrinsic to the cells and is not related to the oxygen/carbon dioxide levels in our culture conditions.

A standard paradigm of cell proliferation requires the recruitment of cells out of the quiesced state (G_0) to begin progression through the cell cycle en route to cell division (3, 13, 23, 24). Upregulation of cyclin D1 protein expression is an important initiating event of cell proliferation. Cyclin D1 combines with cdk4 (or cdk6) to form an active kinase complex that then phosphorylates the tumor suppressor, Rb. This initial phosphorylation leads to an increase in cyclin E transcription. Once cyclin E binds to cdk2 and this complex becomes active, it completes the phosphorylation (and inactivation) of Rb, releasing E2F, an important transactivator of genes such as thymidine kinase, thymidylate synthetase, and dihydrofolate reductase, which are required for DNA synthesis (23, 24, 26).

Both cyclin D1 synthesis and stability are regulated by Ras-dependent pathways (9). In most cells, cyclin D1 has a half-life of ~20 min and is degraded through phosphorylation-dependent ubiquitination via the 26S proteasome (8). Withdrawal of serum leads to a decrease in cyclin D1 transcription as well as a decrease in protein half-life, the result being a drop in cyclin D1 protein levels leading to G_1 arrest. We observed that PMVEC did not become quiescent despite being deprived of growth factors. The increased number of cells in S phase, evidence of ongoing DNA synthesis (as demonstrated by BrdU incorporation), and an increase in cell number all support the conclusion that these cells were not quiesced. By working backwards in the cell cycle, we identified that Rb was hyperphosphorylated (inactive) in PMVEC in contrast to the hypophosphorylated state that was predominant with PAEC. Consistent with our observation that Rb was inactivated in PMVEC, we identified active cdk4 and cdk2 complexes; inhibiting their kinase activity by overexpressing the cdk inhibitors p21^{Cip1/waf1} or p27^{Kip1} led to the hypophosphorylation of Rb with subsequent G_0/G_1 arrest, implicating these complexes in the proliferative phenotype observed. Finally, we demonstrated that cyclin D1 levels were elevated in PMVEC relative to PAEC in low-serum conditions. The sum of these observations suggest that the increased cyclin D1 levels in PMVEC may be the initial event leading to the proliferative phenotype observed. Other possibilities, including the presence of signaling pathways that bypass cyclin D1 and directly activate cyclin E-ckd2 to inactivate Rb (21), should also be considered to explain these findings, however.

Cyclin D1 is considered an oncogene, and increased expression is commonly seen in malignancy, particularly breast cancer (1, 11). PMVEC did not have the characteristics of transformed cells, however, once confluent, they underwent a G_0/G_1 arrest and stopped proliferating. Since cyclin D1 protein expression did not drop, even after cells became confluent, it appears that the G_0/G_1 arrest observed in PMVEC at confluence was due to an increase in the cdk inhibitor, p27^{Kip1}. An important role for increased p27^{Kip1} levels in arresting endothelial cells at confluence has been established for aortic endothelial cells proliferating in 10% serum (4). We have shown that withdrawal of serum is a potent inducer of p27^{Kip1} protein expression in rat and mouse pulmonary artery smooth muscle cells even when cells are nonconfluent (10). Serum withdrawal did not increase p27^{Kip1} levels in nonconfluent PMVEC or PAEC in these studies, however. Only when levels of p27^{Kip1} were increased, either by the establishment of a confluent monolayer or through adenoviral overexpression, did the PMVEC come out of cell cycle. Even though p27^{Kip1} failed to increase in both PMVEC and PAEC following serum withdrawal, the proliferative advantage of PMVEC likely relates to its significantly higher levels of cyclin D1.

The importance of a rapid proliferative response to endothelial disruption in the pulmonary microcirculation may relate to its critical role in gas exchange. The strategy adopted by PMVEC, keeping cyclin D1 protein levels high even when growth is constrained by confluence, makes some biological sense. It is easier to rapidly repair a breached monolayer if the early G_1 cell cycle protein, cyclin D1, does not require synthesis. The upstream signaling pathways that contribute to the increased cyclin D1 levels in PMVEC are unclear, however. Analysis of upstream pathways such as those involved in mitogen-activated protein kinases (17, 18), β-catenin (19), protein kinase A (20), and phosphoinositide 3 kinase (PI3)/protein kinase B (AKT)/tuberous sclerosis complex (TSC) signaling (8), all of which can regulate cyclin D1 protein expression depending on condition and cell type, will be required to determine their relative contribution to the proproliferative phenotype exhibited by PMVEC.
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