Molecular and Cellular Determinants of Lung Endothelial Cell Heterogeneity

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between a stainless-steel hook connected to a force transducer (Grass FT-03; Grass; West Warwick, RI) and a glass hook in the organ bath. These vessel rings were equilibrated in Krebs buffer at 37°C for 30 min with constant bubbling of a premixed gas consisting of 20% O_2, 5% CO_2, and balance N_2. A resting tension of 0.5 g was applied to each ring. After a pre-equilibration period, these pulmonary arterial rings were first constricted with KCl (50 mmol/L) to establish references for comparison. Prior to introducing hypoxia (1% O_2, 5% CO_2, and balance N_2, pO_2 approximately 10 mm Hg) to the organ baths, these pulmonary arteries were preconstricted with 1 μmol/L of phenylephrine.[10] Changes in isometric tensions were amplified (Grass DC pre/amplifier model 7DAF; Grass) and recorded digitally (Polyview Software; Grass-Telefactor; West Warwick, RI).

Results

All three RyR isoforms are expressed in rat pulmonary arteries and in isolated PASMCs (> 99% positive for smooth-muscle cell-specific α-actin) by RT-PCR analysis (Fig 1, *top left*, *A*). The distribution of RyRs in PASMCs as assessed by an immunofluorescence method[8] using monoclonal, nonisoform specific anti-RyR antibodies appears to be diffuse throughout the cytoplasm (Fig 1, *top right*, *B*).

In preconstricted rat pulmonary arteries, the sustained phase of HPV can be prevented by pretreating these vessels with the RyR inhibitors ryanodine (200 μmol/L) or dantrolene (50 μmol/L) [Fig 1, *bottom left*, *C*]. The changes in tension under hypoxia measured at 30 min after the induction of hypoxia (corresponding to the sustained phase of HPV), were − 1.8 ± 4.4% and − 16.6 ± 6.7% of KCl-induced contraction (mean ± SEM) in ryanodine-pretreated (n = 7) and dantrolene-pretreated (n = 11) groups, respectively. In contrast, a vigorous sustained contraction was typically observed in the control group (+ 29.3 ± 3.6% of KCl-induced contraction, n = 15) [$p < 0.001$ vs either RyR inhibitor group]. Inhibitors of RyRs, however, also have a modest suppressive effect on the transient phase of HPV. Peak transient contraction of pulmonary arteries pretreated with ryanodine or dantrolene was 20.5 ± 1.8% and 23.2 ± 2.9% of the KCl response, respectively, vs 34.4 ± 3.1% in the control group ($p < 0.01$ vs either RyR inhibitor group).

In separate experiments, the addition of dantrolene, ryanodine, or a thiol-reducing agent, dithiothreitol (DTT) [1 mmol/L] during the sustained phase of HPV reproducibly reversed the hypoxic vasoconstriction (Fig 1, *bottom right*, *D*), thus implicating the oxidation of RyR regulatory thiol groups during the sustained contraction of pulmonary arteries under hypoxia. Furthermore, addition of the superoxide scavenger nitroblue tetrazolium (500 nmol/L) during the sustained phase of HPV prevented further hypoxic pulmonary vasoconstriction (but did not reverse it), thus suggesting superoxide and/or its downstream metabolites are endogenous activators of RyRs in PASMCs during this phase of HPV.

Discussion

ROS have been found to be paradoxically increased in PASMCs under hypoxia,[6,7] possibly due to the uncoupling of the mitochondrial electron transport chain or other mechanisms. Moreover, the generation of these ROS has been found to be essential for HPV in isolated lung preparations,[11] and scavenging superoxide greatly attenuates the sustained phase of HPV.[12] The cellular targets of ROS in PASMCs have not been identified. Our studies[4,5] suggest that regulatory thiol groups of RyRs could be the downstream targets of ROS in the signaling pathway of HPV, especially during the sustained phase of HPV. Our data thus suggest a link between previous independent observations that ROS[7,6] and RyRs[3,1] have important roles in the signaling pathway of HPV.

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Molecular and Cellular Determinants of Lung Endothelial Cell Heterogeneity*

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Idiopathic pulmonary arterial hypertension is a progressive and potentially fatal disease with a limited number of therapeutic options. Two key lesions

underlie the pathophysiology of this disease. The principal lesion is found in large- and intermediate-sized blood vessels and is characterized by medial and adventitial hypertrophy/hyperplasia, with distal extension of smooth-muscle layers into normally nonmuscularized vessels. The second lesion, found prominently in severe forms of pulmonary hypertension, originates in small precapillary vessel segments, commonly at blood vessel bifurcations. This "plexiform lesion" is a lumen-obliterative lesion comprised, at least in part, of cells that share endothelial cell attributes, but that have lost the "law of the monolayer." Indeed, the endothelial contribution to the (mal-)adaptive response in pulmonary hypertension is becoming increasingly apparent, with evidence that endothelium plays an important role in promoting the vasoconstriction and hyperproliferation of medial and adventitial cell layers in large- and intermediate-vessel sizes, and lumen obliteration in the plexiform lesion. Recent evidence indicates endothelial cells along the pulmonary artery and precapillary segments are phenotypically distinct and may fulfill different roles in these site-specific lesions. Thus, the present review summarizes our current understanding of pulmonary endothelial cell heterogeneity and discusses the potential role(s) of endothelial cell heterogeneity in the pathogenesis of pulmonary hypertension.

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Key words: epigenetic; permeability; phenotype; pulmonary hypertension; lectins

Abbreviations: eNOS = endothelial nitric oxide synthase; FITC = fluorescein isothiocyanate; NO = nitric oxide

Pulmonary arterial hypertension is a vascular disorder that is defined by a sustained increase in BP > 25 mm Hg at rest, or > 30 mm Hg with exercise, in the presence of a low or normal pulmonary capillary wedge pressure (< 15 mm Hg) [for review see [1–5]]. The principal histologic findings of this disorder include medial (smooth-muscle cells) and adventitial (fibroblast) hypertrophy/hyperplasia, with smooth-muscle cell extension into distal blood vessels that are normally nonmuscularized (Fig 1). Endothelial abnormalities have been described in these large and intermediate, precapillary blood vessels.[6] Their morphology becomes characteristic of an "activated" phenotype with some evidence for loss of barrier function, consequently exposing underlying cells to circulating mitogens.[7] The complex of mediators released by endothelium changes from one that promotes vasodilation and quiesces smooth-muscle cell growth, to one that promotes vasoconstriction and increases smooth-muscle cell growth.[6] Thus, there is general agreement that pulmonary artery endothelial cells fulfill an important role in the adaptive response to pulmonary hypertension.

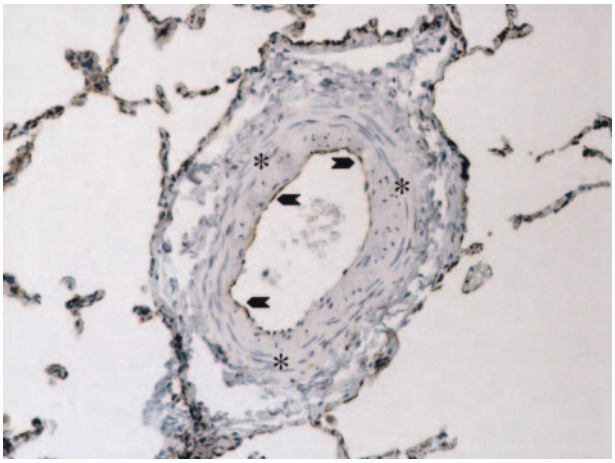

FIGURE 1. The principal lesion in idiopathic pulmonary arterial hypertension involves intermediate- and large-sized pulmonary arteries/arterioles, with medial and adventitial hypertrophy/hyperplasia. Shown is a histologic section of an intermediate-sized pulmonary arteriole from a patient with pulmonary hypertension. The endothelium is resolved by staining (DAB kit; Vector Laboratories; Burlingame, CA) for activated leukocyte cell adhesion molecule (arrowhead). Nuclei are blue. The abnormally large medial wall is shown (asterisks).

In severe pulmonary hypertension, an obliterative, angioproliferative lesion is found most prominently in distal blood vessels.[8–10] This lesion begins in vessels that are approximately 25 μm in diameter, commonly at vessel bifurcations, and can extend proximally into larger precapillary arterioles (Fig 2). Cells in these lesions appear to have an endothelial origin. However, rather than growing in a typical monolayer that defines a patent vessel, they exhibit a proproliferative and apoptosis-resistant phenotype in which the "law of the monolayer" is lost.[11] Attempts to reverse this fixed lesion have been unsuccessful so far, although these efforts have just begun in earnest.

A growing awareness of the principal role endothelial cells play in the development of pulmonary hypertension has reinvigorated interest in fundamental endothelial cell biology, particularly with respect to the stimuli that control their phenotype and function. New data has revealed that endothelial cell behavior varies along vascular segments, in part due to the unique environment of any given segment at any point in time. It is also evident that endothelial cells in extra-alveolar and alveolar segments, respectively, arise from different cell origins during development and, further, that these unique origins imprint a memory that influences cell behavior (for review see Stevens et al[12,13]). Even under similar environmental conditions, extra-alveolar and alveolar endothelial cells possess a distinct phenotype. Thus, understanding the endothelial cell con-

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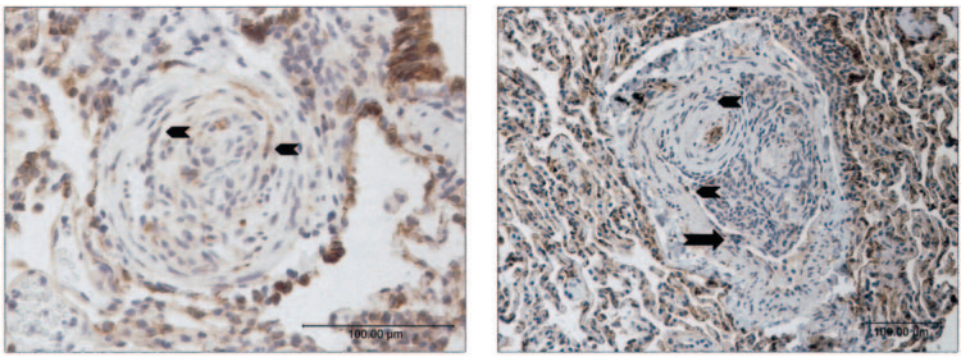

FIGURE 2. Lumen-occluding plexiform lesions are seen in severe pulmonary hypertension. While these lesions may extend into large blood vessels, serial sectioning reveals the initiating locus is typically a small, precapillary arteriole in the range of 25 μm. Shown are histologic sections of plexigenic lesions from two patients. Endothelial cells exhibit reduced staining for activated leukocyte cell adhesion molecule (arrowhead) [DAB kit; Vector Laboratories]. *Right*: A site in the vessel wall where endothelial cells appear to have lost their intimate contact and have grown into the lumen (arrow).

tribution to pulmonary hypertension requires an integrated view of how environmental influences uniquely interact with either arterial or microvascular endothelium to elicit a maladaptive response.

HETEROGENEITY OF LUNG ENDOTHELIUM

Heterogeneity in endothelial cell function is evident along all segments of the lung vascular tree, including the arterial and small precapillary segments that are germane to the pathophysiology of pulmonary hypertension. This heterogeneity is evident in functional studies and in the variable site-specific protein expression patterns (for review see Gebb and Stevens[12] and Garlanda and Dejana[14]). As one example, pulmonary arterial endothelium expresses a greater amount of endothelial nitric oxide synthase (eNOS), and produces more nitric oxide (NO), than capillary endothelium, presumably reflecting the importance of NO in maintenance of low pulmonary vascular tone. Loss of eNOS expression increases both pulmonary vascular reactivity and the susceptibility to developing pulmonary hypertension, whereas eNOS overexpression prevents hypoxic pulmonary hypertension.[15] One phenotypic adaptation to the endothelial cell environment in patients with pulmonary hypertension may[16] or may not[17] be a decrease in eNOS protein. However, even in the event they express enough eNOS protein, they lack bioactive NO because increased arginase activity limits substrate that is available to the enzyme.[17] This limitation in NO bioavailability may increase pulmonary artery pressure, although it is not clear whether the NO limitation is a cause of pulmonary hypertension, or reflects the activated state of endothelium in this disease, especially since there appears to be a global alteration is endothelial production of vasoactive autocoids. Decreased expression of prostacyclin synthase,[18] accompanied by decreased prostacyclin and increased thromboxane production,[19] and increased endothelin-1[20] release—all environmentally induced pulmonary artery endothelial cell adaptations—contribute to the enhanced vasoconstriction that is apparent in pulmonary hypertension.

While decreased NO and prostacyclin, and increased thromboxane and endothelin-1, establish a "vasoconstrictor milieu," they also promote proliferation of subsets of smooth muscle and fibroblasts that reside in the vessel media and adventitia, respectively. This proproliferative stimulus is not likely to be solely due to these autocoids, however, but also to rely on increased access of growth factors from the circulation to the vessel wall.[7] Thus, pulmonary artery endothelial cell barrier function is an important determinant of the vessel wall remodeling that occurs in pulmonary hypertension.

Pulmonary artery and microvascular endothelial cells display different barrier properties that reflect the unique molecular anatomy of their junctional proteins.[12,21–23] In the intact circulation, constitutive fluid filtration is 28-fold and 56-fold greater in pulmonary arterial and venous segments, respectively, than in pulmonary microvascular segments.[23] Capillary endothelial cells may express more vascular endothelial cadherin than their macrovascular counterparts,[14] and some evidence suggests capillary cells also possess epithelial cadherin,[24,25] an adhesion protein typically found in alveolar epithelium. Messenger RNA profiling and Western blot analysis revealed capillary endothelial cells express more neural cadherin and activated leukocyte cell adhesion molecule message and protein than do pulmonary artery endothelial cells,[12] suggesting these junctional proteins may fulfill an important role in the cell-cell recognition and junctional stability of capillary segments.

Pulmonary hypertension is associated with increased fluid flux across endothelium in pulmonary arterial segments, without parenchymal or alveolar edema. These findings are consistent with the idea that endothelial cells in arterial and capillary segments respond differently to circulating inflammatory agonists. There is experimental support for this idea. Oxidant mediated lung injury—including hydrogen peroxide and ischemia-reperfusion—preferentially targets postcapillary segments, whereas mechanical perturbation principally increases capillary endothelial permeability (for review see Gebb and Stevens[12]). Inflammatory agonists that activate Gq proteins initiate cytosolic calcium transitions in endothelial

cells through store-operated calcium entry channels. The activation of store operated calcium entry increases endothelial cell permeability in arterial and venule segments, while typically sparing capillary endothelial cell segments.[21] Based on this available literature, we can understand how the blood chemical environment in pulmonary hypertension may increase pulmonary artery endothelial cell permeability—allowing access of circulating growth factors to the underlying vessel wall—without also increasing capillary endothelial permeability that would promote interstitial and alveolar edema.

Extra-alveolar and Alveolar Endothelium: a Defining Border

These findings illustrate the remarkable endothelial cell heterogeneity that exists all throughout the pulmonary vascular circuit. A part of this heterogeneity is due to the unique tissue and blood microenvironments that exist in different lung compartments. Pulmonary artery endothelial cells, for example, are exposed to mixed venous blood gases and overlay a thick basement membrane that links the endothelium to diverse cell populations in the media. In contrast, capillary endothelial cells are exposed to arterial blood gases and overlay a thin protein matrix tightly associated with type I pneumocytes. Clearly, these and other environmental stimuli influence cell behavior to meet metabolic tissue requirements.

However, different cell environments are not sufficient to explain how, or why, segment-specific behavior is so specialized, and cannot explain how endothelial cells isolated from different segments retain their unique functions *in vitro*, when cell environments are similar. It is becoming increasingly appreciated that macrovascular and microvascular endothelial cells arise from different cell origins during lung development, and that they retain a memory of their vascular origin because they are imprinted to do so.[12] These phenotypically different endothelial cells establish a discernible border in the pulmonary circulation that can be resolved at the extra-alveolar/alveolar border, even in the fully differentiated, adult lung.

The extra-alveolar/alveolar endothelial cell border has been resolved *in vivo* using plant lectins.[26] In the rat lung, *Helix pomatia* recognizes pulmonary artery endothelium, whereas *Glycine max* and *Griffonia simplicifolia* recognize microvascular endothelial cells. Simultaneous delivery of tetramethylrhodamine isothiocyanate-conjugated *H pomatia* and fluorescein isothiocyanate (FITC)-labeled *G simplicifolia* resolves two adjacent endothelial cells with different lectin binding patterns; all endothelium in larger vessels bind *H pomatia* and all microvascular endothelial cells bind *G simplicifolia*, with no evidence of overlap (Fig 3, *A*). *In vitro* studies support these results. Cells in culture retain their lectin-binding patterns, even when they are co-cultured in the same experiment (Fig 3, *B*). Moreover, co-cultured cells tend to segregate and establish a uniquely discernible border between pulmonary artery and microvascular endothelial cells (Fig 3, *C*, and *D*). The mechanism of this unique cell recognition pattern is not understood.

The border between extra-alveolar and alveolar endothelial cells occurs in vessels that are approximately 25 μm in diameter, an important vascular site in pulmonary hypertension. This vessel size is typically poorly muscularized but gains increasing myocyte association as smooth-muscle layers extend distally in pulmonary hypertension. This is also the approximate vessel size where plexiform lesions first appear in severe pulmonary hypertension.[8] Extensive work has indicated the plexiform lesion arises from some type of endothelial cell.[27] It is not clear whether tissue or circulating endothelial cells account for the lumen-occluding lesion. Perhaps more importantly, it is not clear whether cells in the plexiform lesion exhibit a "macrovascular" or "microvascular" phenotype. We addressed this issue using *G simplicifolia* lectin. Human lesions in small-vessel segments possessed cell bundles that interacted with *G simplicifolia*, consistent with a microvascular endothelial cell phenotype (Fig 3, *E*).

The microvascular endothelial cell phenotype has very unique growth properties that may importantly underscore its contribution to pulmonary hypertension (Fig 4). These cells grow much faster than do pulmonary artery endothelial cells,[26] and global expression profiling experiments revealed the up-regulation of a number of proproliferative molecules in these cells, including vascular endothelial cell growth factor. Serum restriction growth suppresses these cells. However, unlike pulmonary artery endothelial cells, serum stimulation during the lag phase of cell growth is sufficient to support subsequent cell proliferation in the 0.1% serum. It will be critical to establish a more detailed understanding of how these cell populations govern their proliferative potential, to better appreciate how microvascular cells (or pulmonary artery endothelial cells) contribute to the plexiform lesion.

Implications of Endothelial Heterogeneity in the Pathophysiology of Pulmonary Hypertension

Idiopathic pulmonary arterial hypertension is a precapillary phenomenon, although it is not exactly clear why. However, the emerging evidence of developmentally early, segment-specific endothelial cell specification provides some insight into how a disease process may be segment specific. Genetic studies have incriminated bone morphogenetic receptor protein II signaling as a cause of pulmonary hypertension in a subset of patients, while dysfunctional transforming growth factor-β and activin receptor-like kinase I signaling has been implicated as a cause of hereditary hemorrhagic telangiectasis and pulmonary hypertension (for review see Budhiraja et al[6]). Activin receptor-like kinase I, in particular, is interesting because its expression is arterial specific during development.[28,29] Such cell specification undoubtedly plays a key role in the highly site-specific nature in which this disease presents and, perhaps, provides insight into the less asked question of why the vascular adaptation does not continue into capillary segments.

While speculative, extra-alveolar and alveolar endothelial cells may fulfill different roles in the pathogenesis of pulmonary hypertension. The principal precapillary lesion

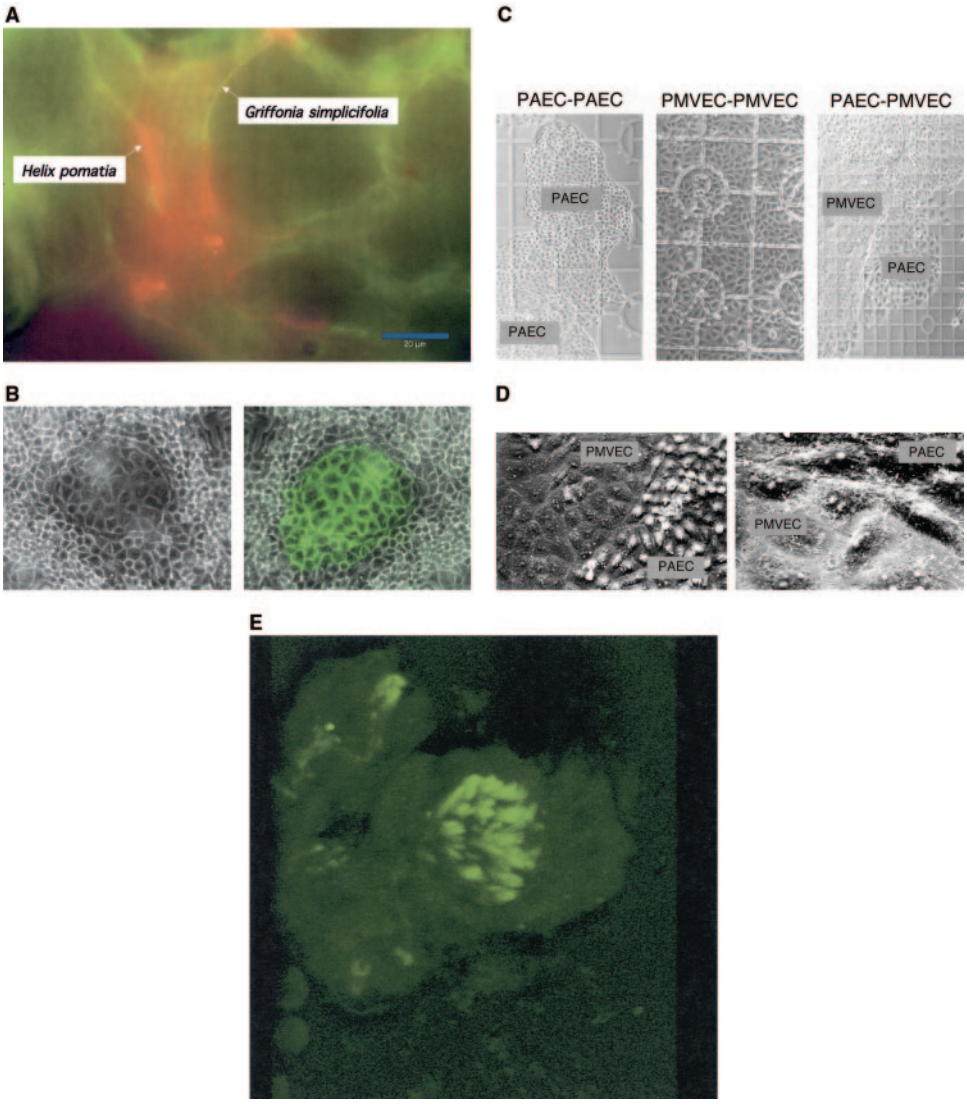

FIGURE 3. Pulmonary arterial endothelial cells (PAECs) are distinguishable from pulmonary microvascular endothelial cells (PMVECs) both *in vivo* and *in vitro*. *A*: Dual labeling of pulmonary artery endothelium using tetramethylrhodamine isothiocyanate-conjugated *H pomatia* and microvascular endothelium using FITC-labeled *G simplicifolia* reveal a site in the intact circulation where different phenotypes interact, at approximately 25 μm in diameter. All upstream pulmonary artery endothelial cells interact with *H pomatia*, and all downstream capillary segments interact with *G simplicifolia* (not shown). *B*: This distinct lectin binding pattern is retained in cultured pulmonary artery and microvascular endothelial cells. Shown is a co-culture experiment where only microvascular endothelial cells interact with FITC-labeled *G simplicifolia*. *C* to *D*: Cell-cell recognition is different among pulmonary artery endothelial cells and microvascular endothelial cells. Cells from different animals were co-cultured on a gridded coverslip and allowed to grow together. Alike cells form a typical cell-cell border that is indistinguishable. However, the pulmonary artery endothelial cell and microvascular endothelial cell border is notable using light (*C*) and scanning electron microscopy (*D*). *E*: Plexiform lesions are comprised of cells that interact with *G simplicifolia*, suggesting a microvascular endothelial phenotype. Human lung specimens from patients with severe pulmonary hypertension were used to analyze plexiform lesions for their ability to interact with lectins. This 70-μm lesion interacts selectively with *G simplicifolia* and not *H pomatia* (not shown), suggesting cells with a microvascular endothelial cell phenotype are present in the lesion.

in resistance vessels may involve an extra-alveolar endothelial cell dysfunction that promotes vasoconstriction and proliferation of cells in the underlying media and adventitia, whereas the plexiform lesion may involve uncontrolled growth of microvascular endothelial cells that ultimately occlude the lumen. If correct, then as the field of pharmacogenetics becomes entrenched in clinical medicine, the drug targets, and sensitivity to pharmacotherapy, will exhibit/possess vascular segment-specific efficacy. Better resolving the site-specific nature of the lesions that are pertinent to pulmonary hypertension will therefore greatly improve our understanding of how to treat this devastating disorder.

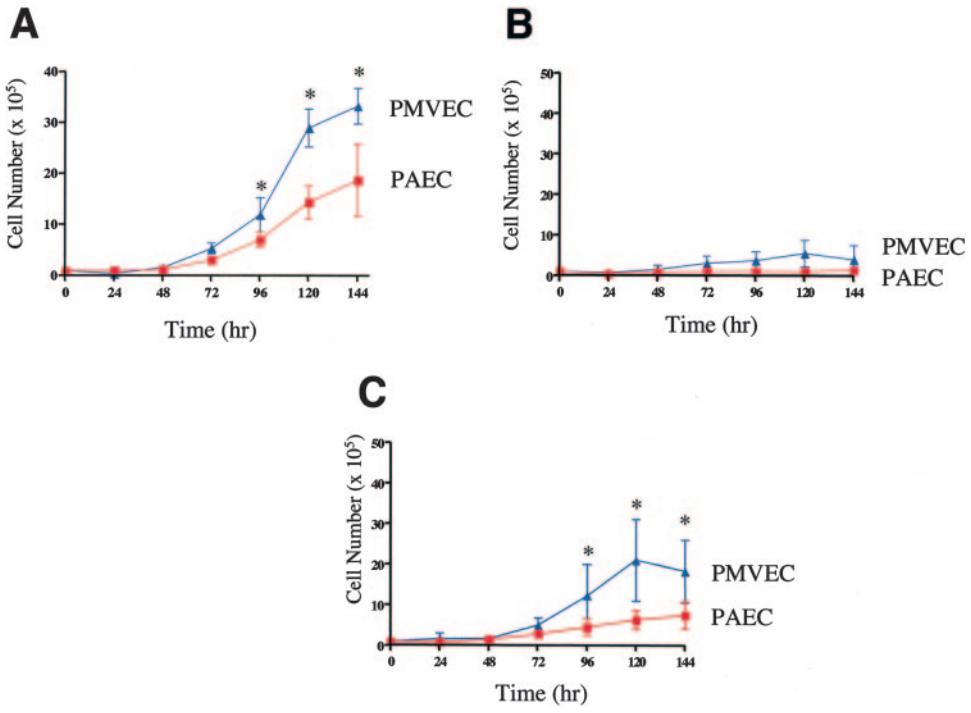

FIGURE 4. Pulmonary microvascular endothelial cells grow faster than do pulmonary artery endothelial cells. *Top left*, *A*: Serum (10%)-stimulated growth curves were constructed in pulmonary microvascular endothelial cells and pulmonary artery endothelial cells, illustrating the greater growth capacity of pulmonary microvascular endothelial cells. *Top right*, *B*: Serum restriction (0.1%) abolished growth in both cells types. *Bottom*, *C*: Cell incubation with 10% serum for 48 h, followed by 0.1% serum, was sufficient to reveal the progrowth program in pulmonary microvascular endothelial cells. See Figure 3 legend for expansion of abbreviations.

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The Extracellular Matrix Microenvironment Specifies Pulmonary Endothelial Cell Identity*

Roles of Tenascin-C and RhoA

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(CHEST 2005; 128:564S)

Abbreviations: EC = endothelial cell; FAK = focal adhesion kinase; MVEC = microvascular endothelial cell; PAEC = pulmonary artery endothelial cell

Endothelial cells (ECs) from the macrovascular (*ie*, pulmonary artery) and microvascular (*ie*, pulmonary capillary) beds are phenotypically and functionally distinct. Consistent with this, pulmonary artery ECs (PAECs) within monocrotaline-treated, hypertensive adult rat lungs interact with an extracellular matrix enriched with tenascin-C, whereas pulmonary microvascular ECs (MVECs) do not. This finding suggests that tenascin-C might contribute to EC heterogeneity within the hypertensive lung. To test this, tenascin-C expression was compared in adult rat PAECs and MVECs cultured on tissue culture plastic, chosen to represent an injured tissue microenvironment. Similar to our *in vivo* findings, tenascin-C expression was significantly greater in cultured PAECs than in MVECs. Since activated focal adhesion kinase (FAK) is required for expression of Prx1, a homeobox gene transcription factor essential for tenascin-C expression, we next examined and found that levels of activated FAK and Prx1 are also higher in tenascin-C–producing PAECs than in MVECs. Furthermore, inhibition of FAK activity not only reduced Prx1 expression in PAECs, but this also resulted in phenotypic change underscored by disruption of cytocortical F-actin. Since tenascin-C modulates F-actin dynamics through suppression of RhoA, (a small guanosine triphosphatase required for F-actin stress fiber formation), we reasoned that RhoA activity and F-actin distribution would differ in PAECs vs MVECs. Indeed, whereas PAECs possessed lower levels of RhoA activity and exhibited cytocortical F-actin, MVECs had higher levels of RhoA activity and displayed prominent F-actin stress fibers. Finally, to determine whether interactions with tenascin-C directly contribute to pulmonary EC heterogeneity, we cultivated tenascin-C–deficient MVECs on exogenous tenascin-C; this treatment suppressed RhoA activity, an event that was associated with loss of F-actin stress fibers in favor of cytocortical F-actin. Collectively, these data indicate that pulmonary EC heterogeneity observed within the hypertensive lung may depend, in part, on the nature of the surrounding extracellular matrix.

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Aberrant Signal Transduction In Pulmonary Hypertension*

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Abbreviations: BMPR2 = bone morphogenic protein receptor 2; ERK = extracellular receptor kinase; PPH = primary pulmonary hypertension

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