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CELLULAR AND MOLECULAR MECHANISMS OF PULMONARY VASCULAR REMODELING

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ABSTRACT

In many organs and tissues, the cellular response to injury is associated with a reiteration of specific developmental processes. Studies have shown that, in response to injury, vascular wall cells in adult organisms express genes or gene products characteristic of earlier developmental states. Other genes, expressed preferentially in adult cells in vivo, are down-regulated following injurious stimuli. Complicating matters, however, are recent observations demonstrating that the vascular wall is comprised of phenotypically heterogeneous subpopulations of endothelial cells, smooth muscle cells, and fibroblasts. It is unclear how specific subsets of cells respond to injury and thus contribute to the vascular remodeling that characterizes chronic pulmonary hypertension. This review discusses vascular development in the lung and the cellular responses occurring in pulmonary hypertension; special attention is given to heterogeneity of responses within cell populations and reiteration of developmental processes.

INTRODUCTION

In several adult tissues (e.g. liver and heart), the cellular response to injury is associated with a reiteration of specific developmental processes. This seems to be true for the vasculature as well. Studies have shown that adult smooth muscle cells (SMCs) responding to injury express genes or gene products (such as

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tropoelastin, fibronectin, tenascin, F31/H19, cytokeratin 8, and extra domain-A fibronectin) characteristic of earlier developmental states (76, 90, 101, 113, 134, 141). In addition, genes such as smooth muscle-specific α-actin, tropomyosin, desmin, and myosin, which are expressed preferentially in adult cells in vivo, are down-regulated when adult cells are stimulated to migrate and divide following injurious stimuli (116, 117). Similar though less extensive reports exist regarding re-expression of developmentally regulated genes by endothelial cells and fibroblasts in response to injury. Complicating matters, however, are recent observations demonstrating that the vascular media is comprised of phenotypically heterogeneous subpopulations of SMCs. It is unclear if all adult medial SMC subpopulations are capable of responding to injury with changes in gene expression and replicative potential, or if the post-injury response is limited to specific subsets of cells within the vessel wall (222). The goal of this review is thus twofold: (*a*) to discuss vascular development in the lung, especially the mechanisms that control growth and differentiation (in an effort to lay the groundwork for understanding the cellular responses and the mechanisms that control them in the setting of pulmonary vascular injury) and (*b*) to discuss the cellular changes that occur in various forms of pulmonary hypertension, with special attention to heterogeneity of responses within cell populations and reiteration of developmental processes (Figure 1).

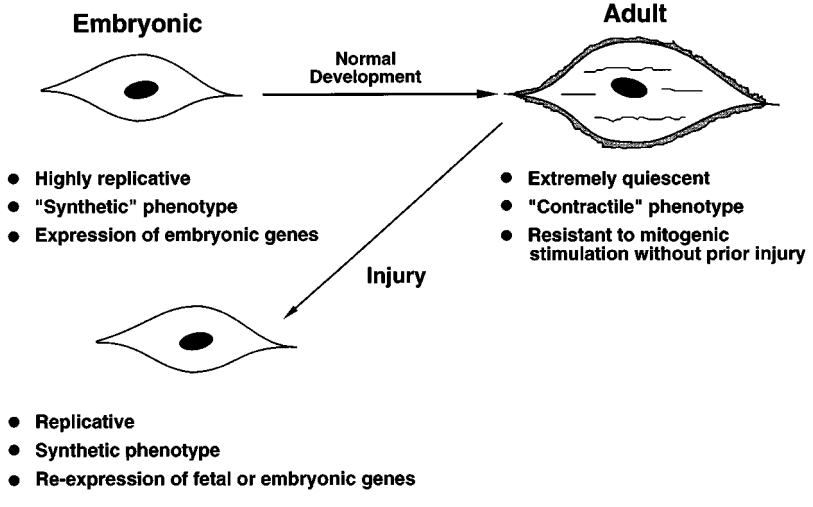


Figure 1 Conceptual figure demonstrating the dramatic changes that occur in SMC phenotype during development. In response to injury, a reiteration of specific developmental processes is observed. SMCs responding to injury express genes or gene products characteristic of earlier developmental states.

DEVELOPMENT OF THE PULMONARY VASCULATURE

The lung is a highly complex organ comprised of more than 40 different cell types (38) that are involved in both respiratory and nonrespiratory functions. Despite its eventual complexity of structure and function, the lung has ostensibly simple beginnings. The lung epithelium originates as paired outpocketings from the floor of the pharyngeal endoderm that expand into mesenchyme derived from splanchnic mesoderm. The epithelial rudiments subsequently undergo a series of repetitive branchings to give rise to the pulmonary tree. Much effort has been directed at defining the mechanisms that regulate normal epithelial pattern formation (termed branching morphogenesis), but much less is known about how the pulmonary vasculature is formed. The development and maintenance of normal vascular structure clearly plays a critical role in lung function, yet several major questions about the regulation of this process in the normal and diseased lung remain unanswered.

Endothelial Cell Replication and Vasculogenesis in the Developing Lung

In the embryonic lung, endothelial precursor cells (angioblasts) initially form a primary vascular plexus within the tissue, which eventually links up to the main circulation, of sixth branchial arch origin, coming from the heart. Studies using intracoelomic chimeric recombinations between quail and chick embryonic lungs have demonstrated that the endoderm seems to control lung vasculogenesis by inducing the emergence of endothelial cells in its associated mesoderm (184). Furthermore, tissue mixing experiments also suggest that vessel formation in the lung, like branching morphogenesis, may depend on the interaction between epithelium and mesenchyme (129, 143, 212). Thus a true reciprocity would exist in which epithelial proliferation and differentiation are induced by an as-yet-unidentified mesenchymal cell population. The induced epithelium (or a subpopulation of cells thereof) in turn produces factors that stimulate endothelial cell proliferation and organization. The endothelium itself may then produce factors to recruit other mesenchymal cells (e.g. SMC precursors) necessary for the completion of vessel structure.

Specific growth factors found in the developing lung, including acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), and platelet-derived endothelial cell growth factor (PD-ECGF), regulate endothelial cell proliferation in vivo and in vitro (81, 84, 99, 216). Another candidate molecule likely to play a major role in lung vascular development is vascular endothelial growth factor (VEGF), a member of the PDGF family that is identical to vascular permeability factor (157) and vasculotropin (190). It is abundant in highly vascularized tissues such as kidney, placenta, and lung. Unlike the FGFs,

VEGF appears to be an endothelial-specific mitogen and is involved in both vasculogenesis and angiogenesis (57). VEGF is detected by antibody staining only in epithelial cells in the glandular stage human fetal lung (232). In situ hybridization studies in the adult rat (157) and guinea pig (19) lung suggest that VEGF expression is localized primarily to the epithelium, although mesenchymal cells may also express VEGF (58).

Two closely related receptor tyrosine kinases are receptors for VEGF: One is the fms-like tyrosine kinase [flt-1 (231)]; the other is the fetal liver kinase [flk-1, the murine homologue of the human KDR gene (197)]. Flk-1 is expressed exclusively in the vascular endothelium in mouse embryos and is the earliest known marker for endothelium and endothelial cell precursors (58, 154, 261). Flk-1 has been detected in the mouse lung as early as day 12.5 (glandular stage) of gestation (279). Beyond the description of the presence of VEGF in the lung and flk-1 in the pulmonary endothelium, however, little is known about the role of VEGF (or other growth factors) in the developing pulmonary vasculature.

Smooth Muscle Cell Replication During Development

The blood vessel initially consists of a single layer of endothelial cells embedded in mesoderm and a scaffolding of extracellular matrix. Development of larger blood vessels (i.e. arteries and veins) from these initial vessels involves recruitment of SMC precursor cells into the region followed by proliferation and then differentiation into mature SMCs. Previous studies in rats have documented the daily replication rates of aortic SMCs throughout development (36). Briefly, SMCs replicate at a high rate ($>$80% per day) throughout embryonic life (Figure 2). At the embryonic-to-fetal transition (between days e17 and e19), the SMC replication rate declines dramatically to approximately 40% per day. During postnatal life, SMCs gradually acquire a quiescent phenotype, reaching a replication rate of $<$0.06% per day in the adult. A nearly identical pattern has been seen in large pulmonary vessels during development (JK Belknap, MCM Weiser, SS Grieshaber, KR Stenmark, RA Majack, submitted for publication). Vascular SMCs therefore undergo a greater than 2000-fold decrease in replication rate from embryonic to adult life.

Available data indicate that rat SMC replication during early development (up to day e17) is autonomous (self-driven) and is not related to secretion of a mitogen. Further, these early embryonic SMCs exhibit poor mitogenic responses to serum and growth factors (36). The dramatic decline in in vivo proliferation observed between days e17 and e19 corresponds with a loss of autonomous growth capacity in SMCs cultured from the great vessels of animals at these time points (36). The switch from autonomous to serum- and growth-factor-dependent growth appears to be caused by the developmental acquisition of a *trans*-acting adult factor capable of suppressing autonomous

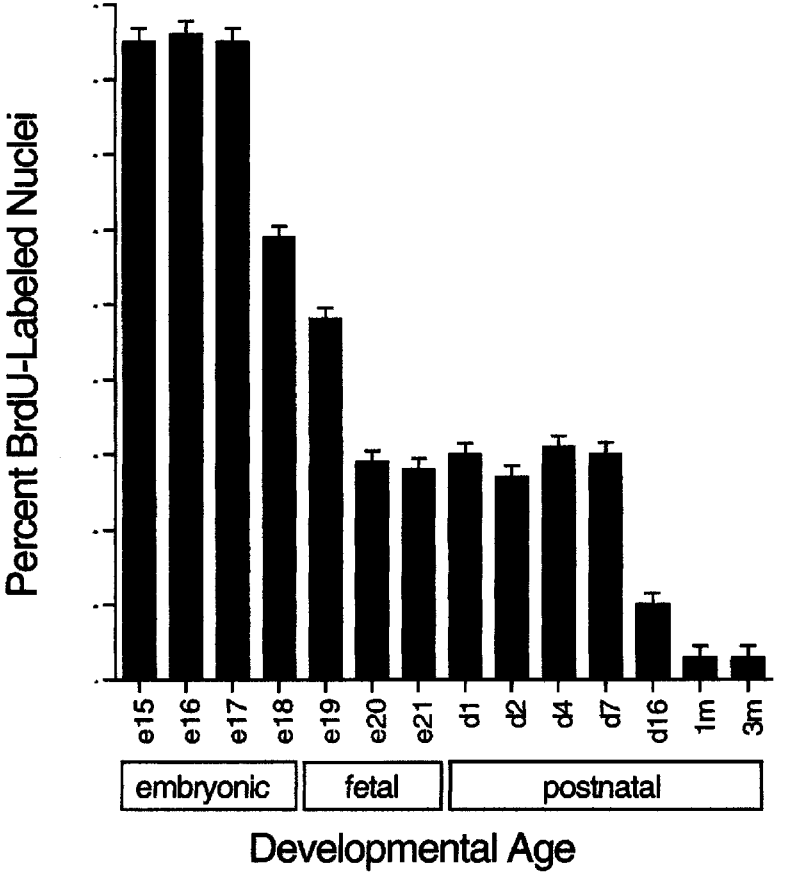


Figure 2 Vascular SMC replication in vivo decreases dramatically during rat pulmonary artery development. Timed pregnant rats were injected with bromodeoxyuridine (BrdU) at the indicated developmental stages, and embryos or fetuses were removed and fixed in 10% formalin. Lung tissue from postpartum rats was processed similarly. Replicating intraparenchymal pulmonary arterial SMCs were identified by BrdU histochemistry. Note that SMC replication rates decrease markedly at the embryonic-fetal transition, remain high until postnatal day 7, and then gradually decline to adult levels (JK Belknap, MCM Weiser, SS Grieshaber, KR Stenmark, RA Majack, submitted for publication).

DNA replication (138). Thus SMC replication during early embryonic vascular development seems to be genetically predetermined and independent of the secretion of mitogens by other cells or tissues.

In the fetal and neonatal periods, in contrast, mechanisms involving growth factors are probably involved in the regulation of vascular cell growth. Recent studies have tested this hypothesis directly (44). SMCs cultured from neonatal calves were compared with cells cultured from adult animals and tested for size differences, serum-stimulated proliferation, susceptibility to senescence, resistance to serum withdrawal, autocrine growth capacity, and responsiveness to growth factors. Neonatal SMCs were significantly smaller in size, grew faster and reached higher plateau density, and were less susceptible to senescence under serum-stimulated conditions than were adult cells. They were also more resistant to induction of a quiescent state, had spontaneous autocrine growth capacity, and were more responsive to mitogens, including IGF-1, phorbol myristate acetate (PMA), or a combination of both. The enhanced growth capacity depended on age at time of harvest.

Neonatal cells also showed an increase in activated PKC activity under basal conditions that may contribute to enhanced responsiveness to IGF-I. The increase was equal to that stimulated by a low concentration (1 nM) of PMA in adult SMCs, a pretreatment that allowed adult cells to be as responsive to IGF-I as untreated neonatal ones. Thus this increased growth of isolated neonatal pulmonary artery SMCs depended on cell size, age at time of cell harvest, IGF-1, and autoactivation of PKC (44).

The factors that drive SMC proliferation during development are largely unknown. Studies have confirmed roles for various growth factors in influencing SMC proliferation in vitro, although the in vivo roles of these factors during development have not been firmly established. Because some of these growth factors play a role in vascular repair and disease, they are likely to play a role in the developing vessel as well. Table 1 lists many of the growth factors, cytokines, and lipids that are produced by vascular wall cells and are known to influence SMC growth and matrix protein synthesis (for review, see 158).

Several vasoactive mediators produced by the vascular endothelium may also play roles in vascular development. Blood flow to the lung is limited during fetal life, and regulation by locally produced vasoconstrictors is important (158). These same vasoconstrictors may exert growth-promoting effects on vascular SMCs, as has been described for thromboxane A2 (100), angiotensin II (ATII) (110), endothelin (24), leukotrienes (183), substance P (168), and 5-hydroxytryptamine (166). Recent studies have suggested potential roles for ATII and endothelin-1 in vascular development.

ATII is a powerful vasoconstrictor in the pulmonary circulation. In addition, angiotensin can stimulate specific intracellular growth events in vascular SMCs

Table 1 Factors regulating SMC growth and matrix protein synthesis

Factors	SMC growth	Elastin	Type I collagen
IGF-I	↑	↑	↑
IGF-II	↑	↑	↑
TGF β	↑/↓	↑	↑
PDGF	↑	↑	↑/↔
Leukotriene C_4(LTC_4)	↑	ND	↑
PAF	↑	↑	ND
IL-1	↑/↓	↓	↑/↓[a]
EGF	↑	↓	↔
Prostaglandins (PGE_2,PGI_2)	↓	↔/↓	↓
Interferons	↓	↓	↓
Protein kinase C activation (PKC)	↑	↓	↓
bFGF	↑	ND	ND
aFGF	↑	ND	ND
Insulin	↑		
TGFα	ND	ND	ND
HB-EGF	↑	ND	ND
PD-ECGF	↔	ND	ND
VEGF (VPF)	↔	ND	ND
Serotonin	↑	ND	ND
Histamine	↑	ND	ND
TNFα	↓	ND	ND
Heparin sulfates	↓	ND	ND

[a]Effects depend on cell type studied; ND, not determined.

(224). It stimulates protein and DNA synthesis, up-regulates c-fos and c-myc expression, and stimulates PDGF-A chain gene expression in vascular SMCs (18, 224, 225). Moreover, ATII selectively increases the expression of α-SM-actin and myosin heavy chain by rat aortic SMCs in culture (269), and it affects the synthesis of extracellular matrix proteins by SMCs (226).

The angiotensin receptor subtypes (AT1a, AT1b, and AT2) are highly regulated during fetal development (82, 268). However, the tissue distribution of angiotensin receptors in the fetus varies considerably, and the fetal lung expresses only the AT1a receptor subtype (228). The expression of angiotensin receptors in undifferentiated fetal mesenchyme also supports a possible role for ATII as a growth and differentiation factor during development (82, 228, 268). The developmental patterns of angiotensin-converting enzyme (ACE) and angiotension I (ATI) expression in the developing lung vasculature were examined recently. Morrell et al (160) demonstrated that ACE expression in developing pulmonary arteries of the rat occurred in a regulated fashion starting with larger proximal vessels as early as day 15 of gestation and spread distally to involve progressively smaller vessels, so that by term all vessels accompanying airways

down to the level of terminal bronchioles expressed ACE. In addition, the distal extension of ACE expression into progressively smaller arteries during lung development was correlated closely with the appearance of markers specific for SMCs in the walls of these vessels.

Moreover, in situ hybridization studies demonstrated expression of ATI mRNA by undifferentiated lung mesenchyme surrounding endothelial structures, beginning one day before the appearance of vascular ACE and α-SM actin in the peripheral lung. ATI mRNA expression was reduced greatly in the differentiated smooth muscle of the histologically apparent tunica media. These results suggest that local production of ATII by ACE, and the expression of the ATI receptor by undifferentiated lung mesenchyme, could play a role in the muscularization of developing pulmonary arteries.

Endothelin (ET) is the most potent vasoconstrictor known (30). Three isopeptides (ET-1, ET-2, and ET-3), encoded by different gene loci, act on two distinct G-protein-coupled receptors (ETA and ETB) with different affinities. Endothelin mRNAs and binding sites are present in the developing rat lung vasculature as early as fetal day 19 (137). In addition to vasoconstrictive effects on vascular SMCs, endothelin stimulates proliferation of vascular SMCs (121, 259). However, its potency as a smooth muscle mitogen is poor in the absence of other growth factors. Endothelin also stimulates pulmonary artery adventitial fibroblast proliferation and chemotaxis (187) and up-regulates fibroblast collagen synthesis (106). SMCs can also produce endothelin, suggesting an autocrine function, and exposure to ATII, arginine vasopressin, or PDGF-AA increases vascular SMC production of endothelin (205). These effects of ET-1 suggest endothelin may participate in the regulation of cardiovascular development and/or homeostasis. Recent studies evaluating homozygous ET-1 null mutation mice confirm that ET-1 is essential to normal development of the heart and great vessels. Edn1 $-/-$ homozygotes displayed cardiovascular malformations, including interrupted aortic arch, tubular hypoplasia of the aortic arch, aberrant right subclavian artery, and ventricular septal defect with abnormalities of the outflow tract (126).

Extracellular Matrix Protein Changes During Development

ECM protein production by all cells in the vascular wall also changes significantly during the course of normal development. ECM not only provides structural integrity to the developing vessel and a lattice to which cells can anchor themselves, but it also provides informational clues that affect a variety of cell processes, including proliferation, migration, differentiation, and the synthesis of other types of matrix molecules. Information encoded in or received by the ECM can be transmitted to the internal domain of the cell (31, 209). The connective tissue elements of the vascular wall are complex and

include molecules from virtually every class of extracellular matrix: elastin, collagens, proteoglycans, and structural and adhesive glycoproteins. The relative proportions of each matrix component appear to reflect either the physical and functional properties expected of a particular vascular segment or the forces to which that vascular segment is exposed. Thus pulmonary vascular protein composition changes significantly with development and with type and location of the vessel studied (155).

For example, elastin is the most abundant protein in major arteries subjected to large pulsatile pressures generated by cardiac contraction (132). Elastogenic progenitor cells, giving rise to at least some of the SMCs that constitute the aorta and other elastic vessels, arise from mesenchyme adjacent to the myocardium (226). Elastogenic activity in these cells, at least in developing chick aorta, appears in an orderly, sequential, proximal-to-distal deployment, beginning nearest the heart and advancing downstream into all nascent elastic vessels. In the developing rat aorta, peak elastin synthesis occurs during late fetal and early postnatal life and remains high through the first postnatal month (147, 163). By two months, tropoelastin expression is virtually undetectable, and no significant tropoelastin is produced by the normal adult vascular smooth muscle cells (147, 192, 245). The developmentally confined period of elastin production suggests that tropoelastin expression is controlled accurately by tissue- and temporal-specific mechanisms, but the precise mechanisms that govern elastogenesis are unknown.

As distance from the heart increases, a progressive decrease in the amount of vascular elastin and a progressive increase in the amount of collagen is observed (41). Mature cross-linked collagen is much stiffer than elastin and thus confers different structural properties to the vessel wall. Collagens are an important family of structural proteins localized in the basement membrane and interstitial spaces of the vascular wall. Many excellent studies have evaluated accumulation of collagen in both systemic and pulmonary vessels during normal development (112, 277). The amount of collagen in a vessel wall depends on both age and site of the vessel studied. Furthermore, the amount of this fibrous protein, like elastin, can be correlated with differences in vascular wall tension that normally occur in different arteries during growth.

Immunohistochemical studies have evaluated the appearance and distribution of various collagen types in the developing pulmonary artery. During embryonic development, the interstitial collagens are deposited in the order of types V, III, and I (155). The amount of types I and V collagen in pulmonary arterial media increases with age. Particularly striking is the relative paucity of type I collagen in resistance-sized pulmonary arteries of the newborn compared with the adult lung (53, 155). Interesting functional implications can be drawn from these studies. Type I collagen is characterized by a high tensile strength; large

quantities of this protein are prominent in tissues that are relatively indistensible. In contrast, type III collagen is present in distensible connective tissues (e.g. the uterus). The relative lack of type I collagen in developing pulmonary vessels may help explain the plasticity observed in the pulmonary arteries in fetal and early neonatal life. Cells composing the vessel wall change rapidly in shape and position during the first hours of life. The vessel walls themselves are remodeled continually with growth. These events may be facilitated by a paucity of type I collagen. However, although present in small amounts, type I collagen appears crucial for the maintenance of vascular integrity during normal development: Mutant mice, deficient in type I collagen, die at about 15 days of gestation with ruptured major blood vessels (124).

Components of the ECM, such as collagen, laminin, fibronectin, and perlecan, are capable of influencing the proliferation, migration, and differentiation of cultured endothelial and smooth muscle cells (59, 89, 91). The distribution of these proteins and the sequence of their appearance have been studied in various tissues during development in attempts to determine whether ECM components play a role in regulating the growth and patterning of blood vessels in vivo. Information on the timing of basement membrane formation during vascular development is crucially important to our understanding of the role the ECM may play in the control of SMC replication, migration, gene expression, and growth responsiveness during development and in response to injury.

Studies of blood vessel formation in the chick chorioallantoic membrane (CAM) have demonstrated that fibronectin is one of the earliest components to be deposited in the vascular basal laminae. The appearance of fibronectin in the perivascular ECM precedes by at least two days the appearance of ultrastructurally identifiable basal laminae, which are not evident until day 6 (9). The location of fibronection relative to the developing blood vessels is consistent with it playing a role in certain critical stages in the formation of capillaries and larger vessels. The presence of fibronectin in the allantois, where the primitive blood vessels are first formed, suggests this molecule participates in the early aggregation of mesenchymal cells to form capillary tubes. As in other embryonic tissues, fibronectin could promote the migration of capillary endothelial cells because it stimulates the migration of vascular endothelial cells in vitro (270).

As fibronectin levels peak in developing arterioles and venules of the CAM, glycosaminoglycans (8) and type IV collagen are detected. The ability of fibronectin to bind to cell surfaces as well as to matrix components such as collagen and proteoglycans (97, 213) suggests fibronectin plays a role in the progressive accumulation and organization of the basal lamina of endothelial and smooth muscle cells. Fibronectin content then diminishes in the basal laminae with time and is usually a minor component of adult vascular basal laminae. The mRNA for fibronectin undergoes extensive alternative splicing that results

in at least ten different fibronectin variants (121). At least two of these peptides appear to be developmentally regulated in vascular SMCs. For example, the aortic media of the 10- to 12-week old human fetus expresses fibronectin protein that binds to monoclonal antibodies recognizing both extradomain A (ED-A) and extradomain B (ED-B) fibronectin sequences. With continued development to 20–25 weeks, the proportion of ED-A antibody-recognizing fibronectin decreases fivefold and that for ED-B decreases twofold. By adulthood, no ED-A- or ED-B-recognizing fibronectin sequences can be found in the vessel media; however, ED-A is seen in SMCs in the normal adult arterial intima (76).

Laminin also appears early in vessel walls although later than fibronectin. It promotes the morphological differentiation of human capillary and venous endothelial cells, cultured in vitro, into capillary-like structures (125) and promotes the maintenance of the contractile phenotype and other differentiated properties of cultured arterial SMCs, which in the absence of laminin tend to lose their microfilaments and assume a less differentiated and more synthetically active phenotype (89). The appearance of laminin in vivo does not seem to inhibit endothelial cell proliferation as it does in vitro.

Type IV collagen is the last basement membrane collagen to appear in the matrix surrounding CAM vessels. It is not present during the earliest stages of vessel development, when endothelial cells are proliferating rapidly, but appears only as endothelial cell division starts to slow (9). Type IV collagen appears in the vascular basal laminae at approximately the same time as sulfated proteoglycans (8). Type IV collagen enables cultured SMCs to maintain a contractile phenotype (90) and thus could be involved in the progressive differentiation that occurs later in vascular development.

Endogenous heparin-like molecules are believed to play critical roles in the control of vascular SMC replication (17, 32, 65, 262), migration (139), and differentiated functions (134, 263). The pattern of expression of perlecan, the predominant basement membrane heparan sulfate proteoglycan (HSPG), during pulmonary and aortic development has been described in studies of the rat (JK Belknap, MCM Weiser, SS Grieshaber, KR Stenmark, RA Majack, submitted for publication; 275). Expression of perlecan mRNA and protein in aortic SMCs was first observed at day e19, a time that marks a dramatic decline in SMC replication rate and a change in growth phenotype (Figure 3) (275). Expression of perlecan message and protein was high throughout fetal and early neonatal life and remained readily detectable in the adult aorta. Several studies suggest the expression of perlecan by vascular SMCs is regulated by apparent developmental age as well as by cellular growth state. The developmental timing of perlecan expression suggests this HSPG may play a determining role in the cessation of SMC replication during vascular morphogenesis and participate in maintaining SMCs in a quiescent state in the adult blood vessel

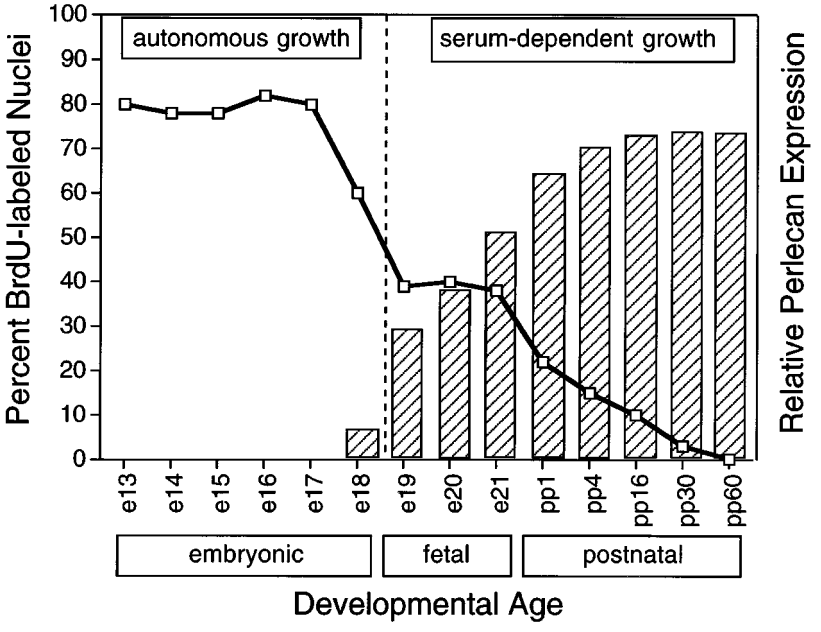


Figure 3 Inverse correlation between perlecan expression and SMC replication during aortic development. During development, aortic SMCs demonstrate very high in vivo replication indices (analyzed as the percent of BrdU-positive cells) throughout embryonic life (to e18) and demonstrate the potential for autonomous (serum-independent) growth in culture. At the embryonic-to-fetal transition, the SMC replication rate decreases dramatically and then more gradually until the adult level of less than 0.1% is reached. This transition point appears to mark a major change in SMCs to a nonautonomous (serum-dependent) growth phenotype (36). Concurrent with the decline in replication during development is a marked increase in the expression of perlecan by nonreplicating aortic SMCs, suggesting that the gradual deposition of a perlecan-rich, growth-inhibitory ECM may prevent SMC replication in the adult, uninjured blood vessel. The line represents percent of BrdU-positive aortic SMCs. Bars indicate perlecan expression by aortic medial SMCs (275).

wall. Alternatively or in addition, perlecan expression by quiescent SMC may reflect the deposition of a protective growth-suppressive and differentiation-promoting basement membrane. Removal of such growth-inhibitory influences may be a major determinant of the extent of SMC replication following injury.

Changes in Contractile Protein Expression During Development

Significant changes also occur in expression of contractile and cytoskeletal proteins during normal development. Morphological studies have demonstrated that the volume percentage of myofilaments in the aorta increases from 5% at

birth to a maximum of 20% at 8 weeks of age. The noncollagenous, alkaline-soluble protein fraction of the vessel wall, which in part represents the contractile proteins, does not change after 10 weeks of age. Thus the net production of contractile protein increases during the first 10 weeks after birth and then remains constant. Concomitant with the increase in actin (a major SMC contractile protein) content accompanying SMC maturation is a switch from predominant expression of non-muscle β-actin by neonatal aortic SMC to predominant expression of smooth muscle (SM)-specific α-actin isoforms by adult SMC (116).

In addition to this shift from non-muscle β-actin to the SM-specific form of α-actin during maturation, the cellular content of tropomyosin, vimentin, and desmin in SMCs increases with age (162, 181). Recently the SM2 isoform of SM-myosin heavy chain (SM-MHC) was found to be expressed only in adult blood vessels, while the SM1 isoform was found to be expressed earlier in development (127, 218). Newborn rat aortic SMCs underexpressing α-actin isoforms exhibit a higher replication rate than do α-actin positive cells, suggesting an inverse correlation between acquisition of the adult phenotype and cell replication. However, during development, contractile and synthetic function of SMCs are not mutually exclusive. SMCs in developing vessels simultaneously express multiple SM contractile proteins, a variety of ECM proteins, and they proliferate (181, 227).

Studies in the developing human aorta have evaluated expression of the regulatory contractile proteins h-caldesmon and calponin, in conjunction with expression of α-SM-actin and SM-MHC (62, 75, 78). Actin and myosin are expressed far earlier in development than caldesmon and calponin, suggesting these latter proteins, because they are involved in the regulation of contraction, may serve as markers of more highly differentiated SMCs. In addition, expression of meta-vinculin, a marker of microfilament-membrane association sites, also occurs later in development in more differentiated SMCs (77).

Substantial differences clearly exist among the proliferative, secretory, and contractile phenotypes of fetal and adult vascular wall cells. Additional differences may exist at the gene and protein level, and these differences may play pivotal roles in the control of SMC replication and differentiation during vascular development. Furthermore, these differences in gene expression could contribute to significant variations in the response of cells to injurious stimuli during vascular development.

Factors Possibly Contributing to Growth Suppression in Adult SMCs

In vivo, adult SMCs are replicatively quiescent and relatively unresponsive to mitogens that stimulate cultured SMCs. Exposure of SMCs to exogenous

mitogens alone, either in vivo or in cultured tissue explants, does not appear sufficient to initiate DNA synthesis, suggesting the presence of potent growth-suppressive mechanisms in the adult vessel wall (222). Intracellular and extracellular factors probably contribute to SMC quiescence in the adult blood vessel wall. Intracellular factors that exist in SMCs include tumor suppressor genes, whose loss-of-function mutations or inactivation (e.g. by binding to viral gene products) can lead to a loss of normal growth control (133). Similarly, the existence of an adult factor capable of suppressing the autonomous growth phenotype of embryonic SMCs has been inferred from heterokaryon studies (138). This factor is probably functionally related to tissue-specific extinguishers such as fibroblast TSE-1 (102), which suppresses liver-specific gene expression in fibroblast:hepatoma fusion cells. Other tissue-specific extinguishers appear to play important roles in regulating the expression of differentiation-specific genes (229). Finally, Blau and colleagues have shown that the 3′ untranslated regions of several muscle-specific mRNAs exert growth-inhibitory, tumor suppressive, and differentiation-promoting activity (203). Their data suggest that the expression of differentiation-specific mRNAs may in turn contribute to the maintenance of the quiescent, differentiated state. A similar situation may exist for vascular SMCs.

Extracellular factors that may contribute to developmental growth suppression in vascular SMCs include growth factors whose disappearance during development might lead to passive growth arrest and growth inhibitors such as those of the transforming growth factor beta (TGF-β) superfamily (114). Components of the ECM also appear to contribute substantially to the control of SMC growth and differentiation. For example, when actively replicating synthetic SMCs are cultured on basement membrane components isolated from the Engelbreth-Holm-Swarm (EHS) mouse tumor (commercially available as Matrigel), they become growth inhibited, activate MAP kinases less efficiently in response to mitogens, and exhibit more differentiated functions (134, 274). Fibronectin appears to facilitate the expression of a synthetic SMC phenotype, while other matrix components may induce the expression of a contractile phenotype and inhibit SMC proliferation (262, 263). Components of the basement membrane or ECM that have been reported to inhibit SMC replication or to promote SMC differentiation include heparan sulfates, laminin, and collagen type IV (263, 282). Other studies have indicated that the developmentally timed expression of perlecan, the predominant basement membrane HSPG, may contribute substantially to the establishment and/or maintenance of SMC quiescence in the mature blood vessel wall and may regulate the expression of certain growth-essential transcription factors (274, 275) (see Figure 3). The mechanism(s) by which perlecan or heparin-like molecules regulate SMC replication and gene expression is not yet clear, but it may include a number of parallel growth-inhibitory pathways (7).

Table 2 In vivo characterization of four SMC populations within the mature bovine pulmonary artery media

	L1-cells	L2-SMCs	L3-i-cells	L3-C-SMCs[a]
Location	Preluminal	Inner-middle	Outer	
Morphology	Small, irregular	Spindle	Thin, spindle	Outer
Cell orientation	Irregular	Circumferential	Circumferential	Thick, spindle
Pattern of elastic	Fragmented	Well-developed	Well-developed	Longitudinal
Lamellae	Particles	Continuous	Continuous	None
Immunobiochemical characteristics				
Immunostaining:				
α-SM-actin	−	+	−	+
SM-myosin	−	+	−	+
Calponin	−	+	−	+
Desmin	−	+	−	+
Meta-vinculin	−	−	−	+
Western blotting:				
150-kDa caldesmon	−	−	−	+
SM-MHCs:				
SM-1	−	+	−	+
SM-2	−	−	ND	+

[a]i indicates cells in interstitial areas; C indicates cells in compact clusters. SM, smooth muscle; MHC, myosin heavychain; and ND, not determined.

Heterogeneity of SMC Phenotype in the Vascular Wall

A growing body of literature is based on both in vivo and in vitro studies documenting the existence of phenotypic heterogeneity within the resident SMC population of a given tissue at a specified developmental stage.

IN VIVO STUDIES The hypothesis that multiple distinct SMC subpopulations comprise the normal mammalian arterial media is supported by previous observations in the systemic circulation. Several laboratories have reported the existence of SMCs of different size, shape, and differentiation state in the mature systemic vessel wall (180, 239, 281). Recent studies have demonstrated that the pulmonary artery is also composed of multiple phenotypically distinct (as defined by cytoskeletal and matrix protein expression) SMC subpopulations (61, 194, 245, 252) (Figure 4 and Table 2). Further, the data demonstrate that these cells progress along different developmental pathways, thus suggesting the existence of distinct vascular SMC lineages (61) (Figure 5).

The observation of multiple SMC subpopulations comprising the arterial media raises an important question as to why this cellular diversity exists. Numerous functions are required of vascular SMCs under both normal and pathologic conditions including contraction, proliferation, and synthesis of matrix proteins. Different SMC functions may require different phenotypes. Accordingly, in

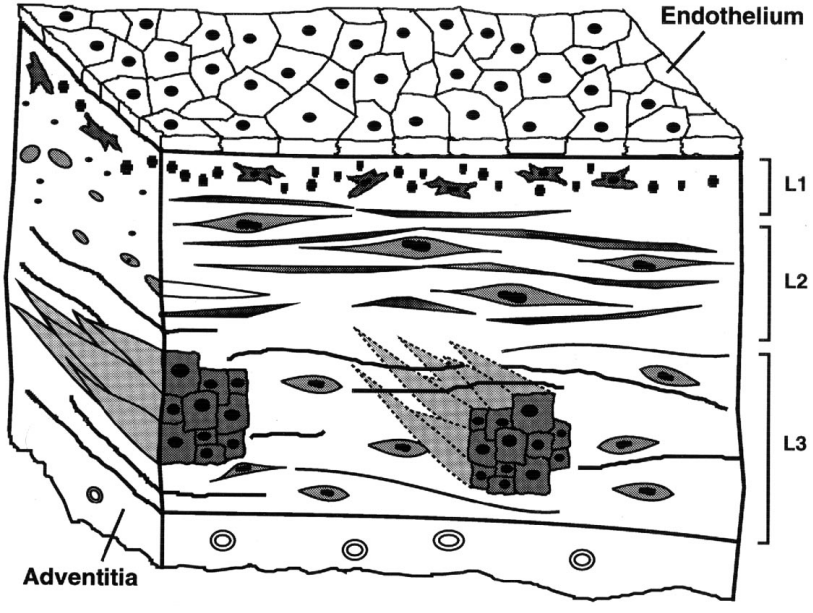


Figure 4 Schematic diagram demonstrating the structural and cellular heterogeneity within the bovine main pulmonary artery. L1, subendothelial media; L2, middle media; L3, outer media. Table 2 describes characteristics of cells located in each layer.

response to pathologic stimuli, some medial SMCs are likely to maintain their normal, mainly contractile functions, whereas others are likely to exhibit elevated proliferation and/or matrix protein synthesis. This diversity in cellular responses would lend itself to a better maintenance of vascular homeostasis after hypoxic injury. Most functions could continue without the severe or fatal compromise that would occur if all (or most) cells shifted simultaneously from one phenotype (contractile) to another (proliferative and/or synthetic).

IN VITRO STUDIES Recent in vitro studies also support the hypothesis that phenotypically distinct SMC subpopulations exist within normal arterial media and exhibit unique and selective responses to the same stimuli. Vascular SMCs of multiple phenotypes have been isolated and maintained in culture over many passages. SMCs distinct in morphology; growth factor requirements; saturation density; and expression of cytoskeletal/contractile proteins, growth factors, and ECM molecules have been derived from systemic vessels from normal rats of different ages and from injured vessels. For example, subpopulations of SMCs have been isolated from the normal arterial media of neonatal rats, which can

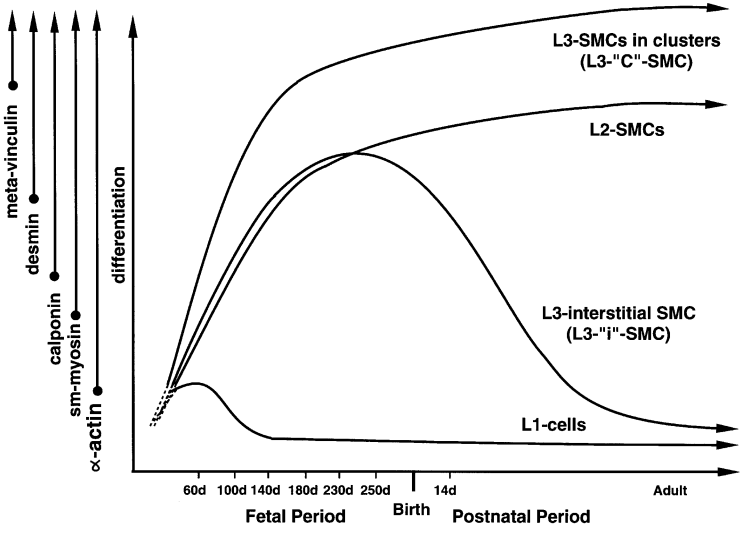


Figure 5 Schematic representation of the pattern of SMC contractile and cytoskeletal protein expression in different cell populations during development. Time is represented along the x axis, and expression of various smooth muscle proteins is represented by the arrows along the y axis. Four distinct developmental patterns are observed. L1-cells are those observed in the preluminal media. L2-SMCs are those identified within the inner-middle media. L3-C-SMCs and L3-i-cells are those observed in the outer media (L3-C-SMCs, in the cell clusters; L3-i-cells, in interstitial areas between the clusters).

grow in PDGF-depleted media, whereas other, simultaneously isolated SMC subpopulations require exogenous PDGF for proliferation (130, 223).

Other studies have shown that a subpopulation of SMCs less sensitive to the growth-inhibitory effects of heparin can be isolated selectively from the adult rat aortic media (13). Most recently, two morphologically distinct SMC populations, isolated from the mature rat aortic media, were shown to differ dramatically in their ionic responses to vasoactive agonists (167). Two stable SMC subpopulations were isolated from human systemic arteries with markedly different growth properties (66). In vitro data from our laboratory (63, 64) demonstrate that multiple morphologically and phenotypically distinct SMC subpopulations can be isolated selectively from the normal mature bovine main pulmonary artery. These SMC subpopulations exhibit dramatically different responses to growth-promoting and growth-inhibiting stimuli. Stable clones of SMCs retaining unique characteristics have been obtained from the systemic circulation as well as from pulmonary arteries (211, 223).

PHENOTYPIC CHANGES IN VASCULAR WALL CELLS DURING THE DEVELOPMENT OF PULMONARY HYPERTENSION

Marked changes in the structure and function of endothelial cells, SMCs, and adventitial fibroblasts take place during the development of chronic pulmonary hypertension. Collectively, these cellular changes determine the alterations in tone, reactivity, and vascular resistance that characterize the chronic pulmonary hypertensive state. Injury-induced changes in cell phenotype are complex and depend on many factors. For example, the age and thus possibly the differentiation state of the cell at the time of injury significantly influence the response observed (199, 245, 251). Further, cellular responses differ remarkably at various locations within the pulmonary circulation. The observed difference may result from the variations in stimuli imparted by pulmonary hypertension at different locations within the vascular bed. Alternatively, cells of a similar type (e.g. SMCs) may exhibit unique characteristics at different locations and thus respond in unique ways to the pulmonary hypertensive stimulus. Finally, even within the endothelial, smooth muscle, and fibroblast populations at a specific location within the pulmonary vascular bed, there is a marked heterogeneity in the cells comprising these general populations. Hidden in this heterogeneity and in the heterogeneity of responses are clues that will help us better define the mechanisms contributing to the structural and functional changes that occur in response to chronic hypoxic pulmonary hypertension. In the following sections, we discuss the response of each cell population in the vessel wall to hypertensive stimuli.

Endothelial Changes Occurring During the Development of Chronic Pulmonary Hypertension

The endothelium creates a nonthrombogenic, semipermeable barrier between the blood stream and all extravascular tissues and fluid compartments in the body. It also influences vascular tone, hemostasis, growth, differentiation, chemotaxis, and the vascular response to injury. Given this important role in such a wide array of vascular functions, it is not surprising that significant adaptations in the structure and function of the endothelial cell take place during the development of hypoxic pulmonary hypertension.

HISTOLOGY/MORPHOLOGY Several studies have characterized the histological changes occurring in the endothelial cells of both large and small vessels in response to chronic pulmonary hypertension (96, 149, 151, 198, 200). In chronic hypoxic pulmonary hypertension, increases in intimal thickness secondary to hypertrophy and hyperplasia is observed in both the endothelial and

subendothelial layers. Focal disruption and lysis of the endothelial cell basement membrane often occur, creating a patchy appearance of microfibrillar material in the thickened subendothelial layer much like that reported in the aorta of hypertensive rats. In addition, the presence of collagen fibers, elastin, and microfibrils in the subendothelial space, internal to the endothelial cell basement membrane, suggests an increase in the production of these proteins by the endothelial cell. In support of this possibility are recent in situ hybridization studies demonstrating that during normal growth and development, endothelial cells contribute to formation of the basement membrane and internal elastic laminae via production and secretion of elastin (248). Under normal conditions, endothelial cell production of elastin appears to be suppressed sometime in late fetal or early neonatal life. In response to injury, the endothelial cell re-expresses tropoelastin mRNA (248). In addition, recruitment of SMCs into this enlarging subendothelial space has also been observed. These cells could also contribute to the protein accumulation that occurs in the subendothelial space in chronic hypoxic pulmonary hypertension.

ENDOTHELIAL CELL MEMBRANE PROPERTIES The structural changes in pulmonary endothelial cells and their plasma membranes observed in pulmonary hypertension are accompanied by alterations in the physiological and metabolic function of the cell. For instance, hypoxic exposure decreases the antithrombotic potential, increases the permeability, impairs normal regulation of vascular tone, promotes release of cytokines and growth factors, and interferes with a variety of plasma membrane–dependent receptor, metabolic, and transport functions of the endothelial cell (20, 171–173).

Hypoxia exerts profound effects on the physical state and composition of pulmonary artery endothelial cell plasma membrane lipids (20, 22). Alterations in the properties of membrane lipids interfere with a number of fundamental cellular membrane functions, including cell cycling, differentiation, proliferation, and transmembrane signal transduction (256, 257). In addition, the activity and kinetics of membrane-bound enzymes and carriers can be affected markedly by membrane lipid composition and fluidity (256, 257). The alterations in endothelial structure and membrane properties observed in pulmonary hypertensive conditions also appear to alter permeability. Stelzner et al (242) have demonstrated increased pulmonary transvascular protein and water leak in rats exposed to chronic hypoxia. Further, hypoxia also increases endothelial cell permeability in vitro (172, 173). These permeability changes in endothelial cultures correlate with the formation of intracellular gaps.

Significant changes in surface coagulant properties and proinflammatory cytokine production by endothelial cells are noted in pulmonary hypertension. For instance, hypoxia suppresses normal thrombomodulin production and induces

procoagulant activity (172). Hypoxia also increases interleukin-1 (IL-1) mRNA levels and IL-1α production in cultured endothelial cells (234). The IL-1 thus produced induces endothelial-leukocyte adhesion molecule-1 (ELAM-1) and increases the expression of intracellular adhesion molecule-1 (ICAM-1) on endothelial cells. Increased expression of ICAM-1 has also been reported in the lungs of hypoxic mice (234). Voelkel et al (272) demonstrated the presence of IL-1α and low-level expression of IL-1α mRNA in lungs from monocrotaline-treated rats, and that treatment of the rats with the interleukin-1 receptor antagonist IL-1rα inhibited the development of chronic pulmonary hypertension. IL-1 could participate indirectly in the vascular remodeling by stimulating platelet-activating factor (PAF) production, c-fos protooncogene expression, and activation of the transcription factor NF-κB (272). These observations suggest that hypoxia induces changes in the endothelial cell, which may lead to prothrombotic or altered interactions with circulating cells that could participate in the pulmonary hypertensive response. Indeed, increased adherence of leukocytes and platelets to the endothelium has been observed in vivo in experimental rats with chronic pulmonary hypertension (96).

PRODUCTION AND RELEASE OF VASOACTIVE MEDIATORS BY THE ENDOTHELIUM

Chronic pulmonary hypertension is associated with changes in the production and release of potent vasoactive substances by the endothelium. Several vasoactive agents possess growth-regulatory properties, and pulmonary vascular remodeling could result from an imbalance of growth-inhibitory vasodilators and growth-promoting vasoconstrictors (246).

Changes in the local production of vasodilatory substances in chronic pulmonary hypertensive states are well described. Decreases in prostacyclin and increases in thromboxane production occur in human primary pulmonary hypertension (PPH) (33). Chronic hypoxia decreases biosynthesis of the potent vasodilator prostacyclin (PGI_2) in freshly excised pulmonary artery rings from neonatal calves (10). A similar reduction in PGI_2 production occurs in endothelial cells cultured from these calves, most likely resulting from inhibition of enzyme activity (cyclooxygenase or prostacyclin synthase) (271). Decreased PGI_2 synthesis by cells in the vessel wall could permit coagulation to occur at the endothelial surface, promote SMC proliferation, and impair local vasodilation, all of which contribute to the chronic pulmonary hypertensive state.

Production of endothelial-derived relaxing factor/nitric oxide (EDRF/NO) by endothelial cells both mediates vasodilation and moderates vasoconstriction in the normal pulmonary circulation (79, 85). However, contradictory reports exist concerning EDRF/NO activity in pulmonary hypertension. Whether NO

production is altered is of some importance because a decrease in the production of this potent vasodilator would contribute to the vasoconstriction augmenting the hypertensive state. Studies of hypertensive human and animal conduit pulmonary arteries have demonstrated impaired endothelial-dependent relaxation to acetylcholine and ADP (5, 49, 208). Recent studies have demonstrated little or no expression of nitric oxide (NO) synthase in the pulmonary vascular endothelium of patients with pulmonary hypertension (70). Similarly, some researchers have demonstrated attenuation of agonist-induced vasodilation in hypertensive lung tissue isolated from chronically hypoxic animals (1). In contrast, others have shown augmented vasodilatory responses to acetylcholine, bradykinin, and platelet-activating factor in hypoxic pulmonary hypertensive calf and rat lungs, suggesting that EDRF/NO activity might be increased in hypertensive pulmonary arteries (12, 179).

Several studies have demonstrated that the competitive NO synthase inhibitor L-NNA causes a marked precapillary vasoconstriction in lungs isolated from rats with pulmonary hypertension, whereas no effect is seen on normoxic vascular tone in normotensive lungs (175). Although other investigators have found no change in baseline vascular tone in response to EDRF inhibitors, chronically hypertensive lungs, at least in some species, exhibit increased levels of both basal and stimulated EDRF/NO activity. Further, based on a study of acetylcholine-induced vasodilation in pulmonary hypertensive calves and on results with ADP in fawn-hooded rats with spontaneous pulmonary hypertension, impaired endothelium-dependent relaxation of isolated conduit pulmonary arteries does not necessarily reflect the vasodilator responsiveness of intact pulmonary vascular beds (5, 179). Additional work is needed to precisely identify the role of EDRF/NO in chronic pulmonary hypertensive states.

Of related interest and particular importance in chronic pulmonary hypertension are the effects of NO and PGI_2 on the proliferative state of the underlying SMCs. Researchers have suggested that the normal low rate of proliferation in SMCs of the tunica media may result partly from the inhibitory action of endogenous vasodilator agents from the endothelium that act by increasing cAMP concentration (PGI_2, PGE_1, and adenosine) or cGMP concentration (NO) (166, 197, 219). For instance, NO-containing drugs are capable of inhibiting stimulated SMC proliferation, although they may do so only at much higher concentrations than might be expected from the basal endogenous release of NO (6, 164, 170). Recent studies have suggested that, depending on the developmental age of the SMC and the dose of NO given, stimulation of growth by NO donors is possible (240). cAMP-stimulating agents may exert greater inhibitory effects on cell proliferation than do cGMP-stimulating agents, while the opposite is true for their effects on vascular tone (166). Further work is needed to define

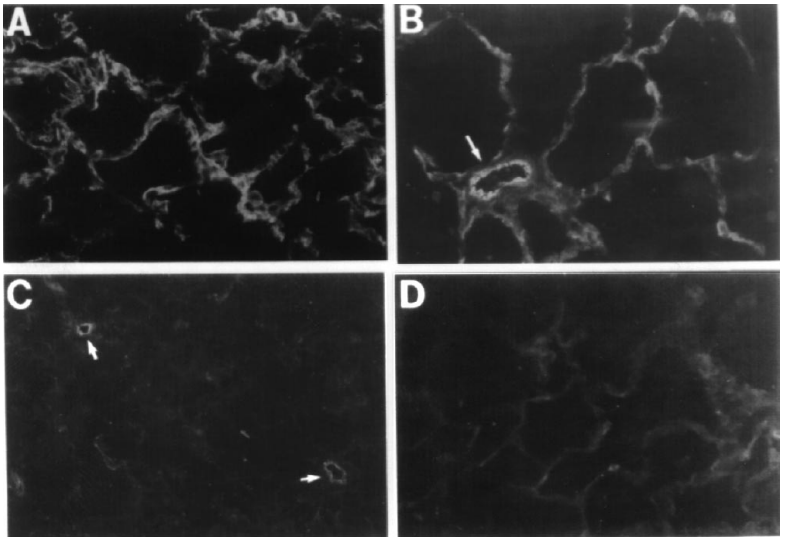
the role of EDRF/NO and PGI_2 in the control of SMC proliferation under both normal and hypertensive conditions.

The endothelial cell is also capable of producing and releasing potent vasoconstrictors, such as endothelin-1 (ET-1) and thromboxane (54, 186). Recent studies have demonstrated increased circulating levels of ET-1 in the pulmonary circulation of adults with both primary and secondary forms of pulmonary hypertension as well as in infants with severe pulmonary hypertension, suggesting a possible role for ET-1 in these conditions (210, 254). Increased tissue expression of ET-1 has also been reported in PPH (72). Recent experimental studies also support the involvement of ET-1 and the ETA receptor in the development of chronic hypoxic and idiopathic pulmonary hypertension (25, 243). ET-1 has been shown to be a mitogen for pulmonary vascular SMCs and fibroblasts, although species differences do exist (93, 187). Thus changes in endothelial ET-1 production occur in chronic pulmonary hypertension and could contribute significantly to vasoconstriction and possibly to remodeling.

In the systemic circulation, ATII formed by the action of ACE on ATI is an important mediator of vascular SMC growth (72) and thus could modulate some of the structural changes associated with pulmonary hypertension. However, substantive evidence of a role for ACE in pulmonary hypertension was lacking until recently. Although inhibitors of ACE attenuate the medial thickening and right ventricular hypertrophy (35), most workers have reported a decrease in whole lung ACE activity during the development of pulmonary hypertension induced by chronic hypoxia (176) or ingestion of monocrotaline (111). These disparate findings have led others to conclude that the beneficial effects of ACE inhibitors on pulmonary hypertension are either independent of inhibition of lung ACE or that down-regulation of lung ACE may be a protective mechanism.

An explanation for this paradox, however, is that whole lung ACE activity does not reflect local pulmonary vascular ACE expression. This result was shown by combining in vivo hemodynamic studies in control and chronically hypoxic rats with measurement of whole lung ACE activity and determination of local pulmonary vascular ACE expression by in situ hybridization and immunohisto chemistry. Morrell et al found that total lung ACE activity was reduced to 50% of control activity by five days of hypoxia and remained low for the duration of the study (159). Immunohistochemistry showed a marked reduction of ACE staining in alveolar capillary endothelium. However, an increase in ACE staining was observed in the walls of small, newly muscularized pulmonary arteries at the level of alveolar ducts and walls. In situ hybridization studies showed increased signal for ACE mRNA in the same vessels (159; Figure 6*a*). Inhibition of ACE by captopril during chronic hypoxic exposure–attenuated pulmonary hypertension and markedly reduced distal muscularization of small

(*a*)



(*b*)

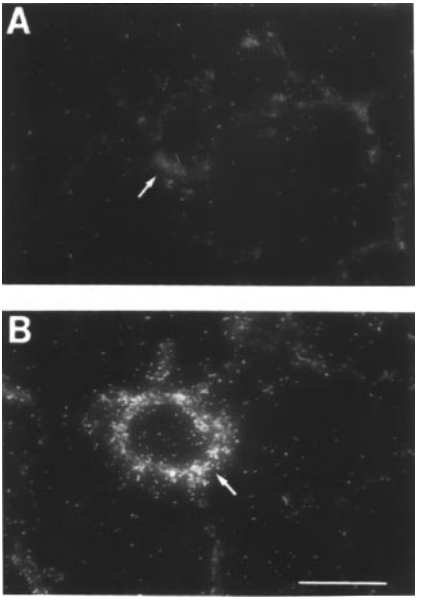


Figure 6 (*a*) ACE immunofluorescence staining in alveolar capillaries in normoxic rat lung and in lungs of rats exposed to chronic hypoxia. Lung sections were incubated with a monoclonal antibody to human ACE (A-C) or control mouse IgG (D). Normoxic lung demonstrated intense staining of the alveolar capillary endothelium (A), which was reduced markedly by day 8 of hypoxia (B and C). ACE staining of the endothelium of small pulmonary arteries was prominent in the hypoxic rats (arrows) (B and C). The staining was specific since virtually no signal was seen in the normoxic lung incubated with control mouse IgG (D). A, B, and D original magnification is 400×; C is 100×. (*b*) In situ hybridization studies of ACE mRNA expression in normoxic (A) and chronically hypoxic (B) rat lung. Virtually no specific signal (white dots) was seen over alveolar capillaries and small pulmonary arteries (arrow) in normal lung (A). An increased signal for ACE mRNA was observed in the walls of small pulmonary arteries at the level of alveolar ducts and walls in chronically hypoxic rats (B). In hypoxic rats, a signal was detected both in endothelial cells and in cells in deeper layers of the vessel wall (B). Bar = ∼50 mm.

pulmonary arteries. In addition, marked longitudinal variation in ACE expression along the normal pulmonary vasculature was demonstrated, and the highest levels were found in small muscular arteries associated with terminal and respiratory bronchioles. Thus local ACE expression is increased in the walls of small pulmonary arteries during the development of hypoxic pulmonary hypertension, despite a generalized reduction in alveolar capillary ACE expression.

In normoxic rats, immunohistochemistry demonstrated that ACE expression was confined to pulmonary vascular endothelium. In the neomuscularized arterioles associated with alveolar ducts and walls in hypoxic rats, the proximity of endothelial and smooth muscle layers (often an incomplete single cell layer) made it difficult to determine whether nonendothelial cells also expressed ACE (159). In situ hybridization studies showed an increased signal for ACE mRNA in these vessels, which often appeared to involve the entire vessel wall rather than the endothelium alone, indicating ACE expression in SMCs or pericytes (Figure 6*b*). Somatic ACE expression has been demonstrated by immunohistochemistry in many nonendothelial cell types, including macrophages, renal tubular epithelium, jejunal epithelium, and neointimal SMCs of the balloon-injured aorta (201, 237). In addition, ACE activity can be detected in fibroblasts and SMCs of the normal rat aorta, albeit at a much lower level than in endothelial cells, and aortic SMC ACE expression is increased in animals with systemic hypertension (4). Increased ACE activity in proliferating vascular SMCs might stimulate their growth in an autocrine and/or paracrine fashion. Study of microvascular SMCs and pericytes isolated from the pulmonary vessels will be necessary to determine whether ACE expression is increased in these cells in chronic pulmonary hypertension.

CYTOKINES AND MITOGENS RELEASED BY ENDOTHELIAL CELLS UNDER CONDITIONS KNOWN TO CAUSE PULMONARY HYPERTENSION The production of several other vasoactive and growth-promoting agents changes in vascular endothelial cells under conditions that cause pulmonary hypertension. Increased PDGF-B chain mRNA levels in lungs of chronically hypoxic rats have been reported (109). In vivo PDGF-B mRNA levels reached a maximum after 1 day of hypoxia, were sustained through day 3, and then decreased gradually over the next 11 days. In addition, PDGF-A mRNA levels were increased but followed a different time course. Hypoxic exposure has also been shown to increase the transcriptional rate of the PDGF-B chain in human umbilical vein endothelial cells (122, 123). Increased shear stress is associated with pulmonary hypertension, and shear stresses are capable of inducing PDGF-A and PDGF-B chain gene expression in cultured vascular endothelial cells (95). Further, stretch of the isolated pulmonary arterial wall, a condition present in larger upstream

pulmonary arteries when downstream resistance is increased, induces PDGF-B chain expression (266). Thus PDGF-A and PDGF-B chain products appear to be involved coordinately and sequentially in the hypoxic pulmonary vascular remodeling process. In a hyperoxic model of pulmonary hypertension, increases in PDGF-B and its receptor ligand have also been found and may be involved in the early stimulation and proliferation of precursor muscle cells (192).

VEGF and its receptors may also play an important role in lung vascular remodeling by functioning as an hypoxia-inducible angiogenic factor (236). VEGF mRNA levels increase dramatically within a few hours of hypoxic exposure and return to background levels when normal oxygen supply is resumed. Tuder et al (267) assessed the distribution of VEGF and of VEGF mRNA in lung tissue of chronically hypoxic rats. The VEGF mRNA and protein were increased in the hypertensive compared with normal lung. Transcripts for the VEGF receptors KDR/Flk and Flt were also increased in chronically hypoxic lungs, and the increase of VEGF receptor expression coincided with the first histologic evidence of vascular remodeling. Increases in bFGF production have also been demonstrated in hypoxic endothelial cells (3).

These observations suggest a remarkable capacity for the endothelial cell to influence local vascular structure and function through the production and release of potent mitogens. As indicated by differences in ET-1 and PDGF production by pulmonary artery versus systemic endothelial cells, however, caution must be taken in extrapolating information from experiments done with systemic cells to the pulmonary circulation.

INJURY-INDUCED CHANGES IN PROTEIN PRODUCTION BY ENDOTHELIAL CELLS

Endothelial cells, in response to hypoxia, are also capable of increased synthesis of several classes of ECM proteins. For example, human umbilical vein endothelial cells have increased mRNA levels for thrombospondin, plasminogen activator inhibitor-1, laminin, and fibronectin in response to hypoxia, while production of actin and von Willebrand factor mRNAs are unchanged (136). These responses appear to be distinct to hypoxia and not the result of either a common stress or a nonspecific response to stress because heat shock produces a much different protein synthetic response than hypoxia.

Of particular importance in chronic pulmonary hypertension may be changes in endothelial cell proteoglycan production. Chronically hypoxic endothelial cells produce and secrete less HSPGs than do normoxic cells (16). Further, experiments in which endothelial cells were exposed to high shear conditions again demonstrated decreased production of the growth-inhibiting HSPGs (16). Thus certain conditions associated with chronic pulmonary hypertension may impair the ability of the endothelium to produce important growth-restrictive

matrix molecules. These observations are consistent with the inverse correlation between perlecan expression and SMC proliferation described during development (see Figure 3).

Changes in Smooth Muscle Cell Phenotype During the Development of Chronic Pulmonary Hypertension

The severity of chronic hypoxic pulmonary hypertension is determined, at least in part, by the extent of structural changes in the medial compartment of the pulmonary arterial wall. These changes include SMC proliferation, hypertrophy, and matrix protein deposition. Blood-borne and locally produced mitogens, hypoxia, and mechanical stress are the stimuli that collectively drive these cellular responses by activating a cascade of intracellular signaling mechanisms, including tyrosine kinases, calcium (Ca^{2+}), and protein kinase C (PKC), that promote SMC growth and/or matrix protein synthesis. Synergy among these stimuli and the resulting cross-talk among signal transduction pathways augments the extent of vascular changes. Susceptibility to these stimuli is enhanced when inhibiting mechanisms such as endothelial barrier function, local production of heparan sulfates, and prostacyclin- and NO-induced increases in cyclic nucleotides are impaired. Intrinsic (i.e. developmental, genetic, and acquired) differences in growth and matrix synthetic capacity and local and regional phenotypic heterogeneity of pulmonary artery SMCs also regulate the pattern of remodeling in the tunica media in response to chronic hypoxia.

HISTOLOGY/MORPHOLOGY Common to all forms of human pulmonary arteriolar hypertension are increases in the thickness of the medial layer of normally muscular arteries and an extension of muscle into smaller and more peripheral vessels (153). These features are found in a variety of clinical conditions but especially in those associated with chronic hypoxia such as chronic obstructive pulmonary disease, pulmonary interstitial fibrosis, kyphoscoliosis, central hypoventilation, cystic fibrosis, and residence at high altitude. Detailed characterization of the changes in SMCs induced by chronic hypoxia, hyperoxia, and monocrotaline have been examined in numerous animal studies (for review, see 153).

Meyrick & Reid (149, 152, 204) described the SMC changes of both the proximal and distal pulmonary vasculature and reported that both the timing and the nature of the responses differ. In the proximal pulmonary arteries of adult rats, the media thickens due to hypertrophy and hyperplasia of the individual muscle cells and an increase in the synthesis and deposition of ECM proteins, including elastin and collagen. In the distal vasculature, the muscularization of previously nonmuscular arteries (so-called extension) is probably brought about by a differentiation and hypertrophy of intermediate cells and pericytes

already in the wall (153, 204). Both types of cells proliferate and acquire a more SM-like appearance.

Using hyperoxia as a stimulus to induce pulmonary hypertension, Jones et al have shown that interstitial fibroblasts are recruited to the vessel wall and undergo a phenotypic switch to the synthesis of proteins producing contractile components (104). These cells produce a network of elastin, which appears to induce the formation of a new elastic lamina between the muscle layer and the endothelium. This internal lamina is not as complete as in normal muscular arteries, so that now the endothelial cell and new muscle cells form frequent contacts. These contacts are different from those observed in normal muscular pulmonary arteries, suggesting that a close and perhaps different communication system may exist between these cells in the newly muscularized vessels.

If the injurious stimulus occurs at or around the time of birth, different cellular responses are noted than if pulmonary vascular injury occurs later in life (87, 245, 251). In newborns dying in the first 36 h after an hypoxic event, the peripheral pulmonary arteries show an extremely thick-walled fetal-like structure (87). Similarly, in pigs exposed to hypoxia from the moment of birth, and calves exposed to hypoxia within 24 h of birth, endothelial and smooth muscle cells retain their fetal appearance at 3 days of age (2, 52, 83). In the case of the calves, this observation correlates with the maintenance of high pulmonary artery pressure, similar to that in fetal life. SMCs of all animals exposed to hypoxia during the first week of life demonstrate an increase in the concentration of myofilaments. In the large arteries the abluminal SMCs show a greater increase in myofilaments than do the abluminal cells. This response is rapid and dramatic in neonates but requires a much longer period of exposure to elicit in older animals. In addition, a marked increase in connective tissue is noted in the neonatal SMCs and vascular wall, and it has been postulated that this increase in connective tissue synthesis and accumulation helps the vessel remain in a contracted or restrictive state.

CHANGES IN SMC PROLIFERATION DURING THE DEVELOPMENT OF PULMONARY HYPERTENSION As described above, rapid SMC proliferation characterizes normal vascular development. Once morphogenesis is complete, however, the medial SMC replicative index decreases to very low levels and remains so under normal conditions. This tight control over SMC proliferation appears to be perturbed in hypertension. For example, significant increases are seen in the [^{3}H]-thymidine labeling index of pulmonary artery cells in adult rats exposed to hypoxia or hyperoxia (103, 150). Concominant morphometric studies of these vessels, however, suggested that hypertrophy, in addition to hyperplasia, of the SMCs was present. Thus the medial thickening of the hilar pulmonary artery from hypoxia-exposed adult rats may be caused in part by polypoid

hypertrophy rather than pure hyperplasia. This theory is consistent with studies in systemic arteries in rats, indicating that SMCs are capable of two in vivo growth responses: hypertrophy or hyperplasia (182).

To determine whether the dramatic increase in medial thickening observed in neonatal hypoxia-induced pulmonary hypertension was the result of hypertrophy or hyperplasia, Orton and colleagues (14, 177) showed that the bromodeoxyuridine (BrdU)-labeling index of pulmonary artery medial cells in hypoxic calves was much higher than that found in control calves. Even in control calves the labeling index was at least tenfold higher than the thymidine-labeling index reported for the medial cells from normal adult rats, a difference probably due to normal smooth muscle growth that is still occurring in the newborn period.

Medial cells from the pulmonary artery of control calves had a high fraction of tetraploid (4N) cells and a low fraction of BrdU-positive cells compared with medial cells from hypoxia-exposed calves. This observation suggested that at least a portion of the 4N cells were stable tetraploid cells rather than cells simply traversing G2 in the cell cycle and that polyploid hypertrophy existed or was developing in the media of control calves. This conclusion is consistent with previous findings showing that medial thickening during normal development in the aorta is associated with an increase in the fraction of large polyploid cells (182). This normal developmental pattern is interrupted by hypoxic exposure and/or hemodynamic stress in the immediate neonatal period, and the subsequent acquisition of large polyploid cells is not observed. The hyperplasia of the newborn in response to stress stands in contrast to the hypertrophy observed in the adult.

An interesting observation in the neonatal calf model shed some light on these findings. Botney et al demonstrated a steady increase in TGF-β mRNA in the pulmonary artery following a normal birth. In those animals with hypoxia-induced pulmonary hypertension, no increase in TGF-β was seen (29). TGF-β inhibits SMC growth (under all conditions tested) in neonatal vascular SMC and induces hypertrophy (RA Majack, personal communication). Thus increases in TGF-β in the normal neonatal pulmonary artery could participate in the cessation of proliferation and the increase in ploidy normally occurring after birth. An absence of TGF-β could contribute to the excessive or continued proliferation observed in large neonatal pulmonary arteries.

CONTROL OF SMC PROLIFERATION IN PULMONARY HYPERTENSION Many factors contribute to the suppression of SMC growth in vivo. Access to blood-borne mitogens, coagulant proteins with proliferative effects, platelet products (including PDGF, serotonin, TXB-A2, and ATP), and inflammatory mediators is normally restricted. Under basal conditions, vasodilatory substances such as prostacyclin and NO are produced that exert antiproliferative effects on adjacent

pulmonary artery SMCs. Endothelial-derived heparan sulfates also exert an inhibitory effect on SMC growth mediated, at least in part, by inhibiting the PKC pathway (195).

Collectively, these inhibitory mechanisms counterbalance an elaborate cascade of pro-mitogenic signaling pathways present in pulmonary artery SMCs. Activation of cell surface receptors and their associated tyrosine kinases and phospholipases (C-gamma, A2, or D) leads to the generation of lipid second messengers [e.g. inositol triphosphate (IP_3), diacylglycerol (DAG), and free fatty acids (FFA)] (118, 169). IP_3 promotes mobilization of intracellular Ca^{2+} stores (79). DAG is an endogenous activator of PKC (169).

An array of other lipids can also stimulate PKC (131, 230). Intracellular Ca^{2+} concentration can also be increased by direct or indirect activation of Ca^{2+} channels that span the plasma membrane. There is almost certainly important cross-talk (and resulting synergy) between the Ca^{2+} and PKC signaling pathways. Ca^{2+} is a cofactor for the classical family of PKC isozymes (169). Increases in intracellular Ca^{2+} can directly activate PKC (94), and PKC-mediated phosphorylation regulates Ca^{2+} ion channel activity (67). Activators of the PKC pathway promote many of the same cellular responses (e.g. contraction, hypertrophy, and proliferation) observed in response to pulmonary hypertensive stimuli (46, 178, 241).

Ca^{2+} and PKC-induced protein phosphorylation initiates a cascade of events resulting in increased DNA synthesis and cell proliferation (169, 233). Identified phosphorylation targets include MAP kinases, K and Ca^{2+} channels, Na/H antiporter, heat shock proteins, and mitogen receptors (98, 156, 238). By activating gene regulatory proteins, these signaling mechanisms can also up-regulate the synthesis of proteins (including polyamine transport proteins, mitogens, receptors, mitogen-binding proteins, and transcription factors) that lead to additional SMC growth (23, 86, 235).

Chronic hypoxia and mechanical stress are two stimuli important to the development of pulmonary hypertension. Both stimulate ion channels (seconds) and key signaling mechanisms early (minutes) and increase growth factor expression later (hours). These effects may stimulate growth directly or they could promote cell proliferation indirectly by enhancing responsiveness to other mitogens (so-called permissive effects). Salvaterra (215) and Cornfield (37) have found that hypoxia causes Ca^{2+} influx in pulmonary artery SMCs. Whether this increase in Ca^{2+} contributes to SMC growth is not yet known. Dempsey has reported that hypoxia stimulates PKC to a small extent in pulmonary artery SMCs, which does not by itself lead to cell proliferation but may increase proliferation of growth factors, such as IGF-1, which synergize with even slight activation of PKC (45–47). Gillespie and coworkers (86) have found that hypoxia increases polyamine transport in pulmonary

artery SMCs. Increased intracellular polyamine levels are important in cell growth, and this hypoxia-induced effect could alter SMC response to other mitogens. In other cell systems, O^2 radicals (perhaps produced in the settings of increased or reduced O^2 tension) increase intracellular Ca^{2+} and activate and then inactivate PKC (80, 202). Finally, Langelben et al (128) have detected increased ET-1 expression by pulmonary artery SMCs upon exposure to hypoxia.

Emerging evidence suggests PKC and Ca^{2+} may be important in the transduction of signals associated with mechanical stress (214). Komuro et al (119) have identified a potential role for PKC in the myocyte hypertrophic response to mechanical loading. Others have described early stretch-induced calcium influx (21) and a later increase in PDGF expression (276) in pulmonary artery SMCs. These mechanical stress-induced effects will likely be found to activate PKC, augment responsiveness to other growth-promoting stimuli, and stimulate proliferation of pulmonary artery SMCs. Therefore, PKC and Ca^{2+} signaling pathways appear to be uniquely important in the regulation of pulmonary artery SMC proliferative responses to hypoxia and mechanical stress. How activation of these pathways leads to gene expression is still unclear, but hypoxia-inducible factor-1 and the shear-stress response element are likely important (145, 206).

Local vascular injury (secondary to hypoxia, mechanical stress, and inflammation) also induces the local expression and release of autocrine and paracrine factors that can change the responsiveness of local SMCs to hypoxic stimuli. Using in situ hybridization and immunohistochemical techniques, researchers have demonstrated many growth factors, including IGF-I, IGF-II, TGF-β, EGF, and bFGF, to be locally synthesized in the media or adjacent compartments of the pulmonary arterial wall during the development of pulmonary hypertension (3, 26, 29, 73, 109, 188, 189, 259, 265). As noted above, cultured vascular cells, including endothelial and smooth muscle cells, release PDGF and/or ET-1 in response to hypoxia. Inflammatory mediators (e.g. IL-1) released in response to hypoxia by endothelial cells enhance the expression of bFGF in nearby SMCs (69). Once present, growth factors such as PDGF-B can also initiate the autocrine production of complementary mitogens such as IGF-I, with which they interact synergistically (42). Similarily, Majack et al (140) have demonstrated that TGF-β stimulates increased PDGF-A expression in vascular SMCs. Many of these up-regulated growth factors (e.g. PDGF, bFGF, and ET-1) stimulate PKC activation and can augment responsiveness to other growth factors (e.g. IGFs) and allow proliferation to occur in response to local environmental changes such as hypoxia (45, 46). These findings also emphasize the potential impact that synergistic interactions between different growth stimuli can have in pulmonary vascular remodeling (43–45).

CHANGES IN MATRIX PROTEIN SYNTHESIS BY SMOOTH MUSCLE CELLS DURING THE DEVELOPMENT OF PULMONARY HYPERTENSION Marked increases in ECM protein synthesis by SMCs in vivo in pulmonary hypertension have been well described (112, 147). Studies in chronically hypoxic adult and neonatal animals from several species uniformly demonstrate increases in the production and accumulation of collagen and elastin in the media of conducting pulmonary arteries. In the adult rat, substantial and rapid increases in the relative rates of connective tissue protein synthesis in the explants of main pulmonary arteries have been demonstrated (191, 207). Within the first few days after blood pressure elevation, relative protein synthesis by vascular medial cells is modestly increased (1.7-fold), yet marked increases in the relative synthetic rates per vessel of collagen (8-fold) and insoluble elastin (9-fold) have been observed. These results have demonstrated a shift toward a greater percent of total protein synthesis devoted to collagen and elastin synthesis in hypertensive SMCs.

Similar findings have been reported in neonatal animals with severe hypoxic pulmonary hypertension (39, 51, 53, 148, 244, 252). Lobar pulmonary artery tissue from hypertensive calves demonstrated an approximately fourfold increase in elastin synthesis and steady-state elastin mRNA levels compared with control vessels. Further studies showed an increase in collagen content, a two- to threefold increase in collagen synthesis, and a twofold increase in the steady-state collagen type I and IV mRNA levels in hypertensive lobar pulmonary arteries. As was observed in the adult rat, no changes in elastin or collagen synthesis were observed over the same time period in the thoracic aorta of the animals. In contrast, SMCs in pulmonary veins demonstrated a marked decrease in elastin and collagen production, demonstrating a regional heterogeneity of the SMC response to chronic hypoxia (185). SMCs cultured from the pulmonary artery of hypertensive animals continued to synthesize increased amounts of elastin and collagen and had increased mRNA levels for elastin and collagen (147). These increases in matrix protein production and mRNA levels persisted through several cell passages. Thus significant changes in the synthetic capability of both adult and neonatal pulmonary artery SMCs appear to occur in response to pulmonary hypertension.

Additional studies were performed with in situ hybridization techniques to determine the spatial distribution and relative concentrations of tropoelastin mRNA production by SMC in intralobar pulmonary arteries during the development of neonatal pulmonary hypertension. Initial studies demonstrated marked differences in the distribution of cells producing elastin in the pulmonary arteries of neonatal calves with pulmonary hypertension compared with controls (194). In 15-day-old control animals, the in situ hybridization signal for tropoelastin mRNA was localized in the inner third of the media. In hypertensive vessels, hybridization signal was observed throughout the entire media of

the vessel, and the strongest signal was seen in the outer media. Based on these findings, it was initially hypothesized that SMCs in the outer vessel wall were rapidly recruited into an elastin-producing phenotype during the development of pulmonary hypertension. This could have been due to an increase in the responsiveness of these cells to local humoral or mechanical stimuli. Alternatively, the pattern of tropoelastin gene expression could have been due to a persistence of a fetal pattern of gene expression that was maintained in the face of continued high pressures in the pulmonary circulation.

We found that in the late-gestation fetus, tropoelastin mRNA was expressed by cells throughout the media of the lobar pulmonary artery, but the relative signal strength varied within the vessel wall (252). The strongest signals were detected in the outer medial SMCs, which were separated by dense foci of tropoelastin mRNA negative cells. In normal animals after birth, all of the lobar pulmonary artery gradually thinned, and the pattern of tropoelastin expression was altered markedly. Serial examination of the arteries demonstrated a gradual decrease in signal intensity for tropoelastin mRNA beginning at the outer medial wall and then progressing toward the lumen. At maturity, no consistent hybridization signal was observed in medial SMCs of the lobar pulmonary artery. In the hypoxic calf, however, these age-dependent changes in pulmonary vessel morphology and tropoelastin expression did not occur. Instead, the fetal and early neonatal pattern of tropoelastin expression and wall thickness persisted after 1, 3, 7, and 14 days of hypoxic exposure (252).

Studies in human PPH suggest that this pattern of response is unique to the neonate and is not observed in adult forms of pulmonary hypertension (27). However, the activation or re-expression of tropoelastin by at least some SMCs in the adult vessel wall may represent, at least in part, a reversion to a more fetal or neonatal phenotype of the SMC.

Changes in ECM production, including increased vascular tropoelastin and type I procollagen synthesis and deposition (107, 264), have also been noted in monocrotaline-induced pulmonary hypertension. Re-expression of tropoelastin by endothelial cells has been observed in adult animals as an early and reliable marker for vascular injury. As discussed above, endothelial cells produce elastin early in vascular development although the elastin gene is suppressed in late fetal or early neonatal life.

Using in situ hybridization to identify the vascular compartments responding to monocrotaline injury, Tanaka et al (259) found that active remodeling was induced in two separate and discrete vascular compartments–along the vascular lumen where injury occurred and at the medial-adventitial border distant from the site of injury. The nature of the response differed by location: Tropoelastin expression was found near the lumen, and procollagen expression was found in the medial-adventitial area. Total TGF-β protein was fourfold higher in

remodeling lungs compared with controls, and gene expression for all three isoforms of TGF-β co-localized with regions of active tropoelastin synthesis along the vascular lumen but not with procollagen gene expression (259).

The above studies suggest that increased local production of autocrine or paracrine factors contributes to changes in smooth muscle ECM production (50). Mecham et al (149) have described a soluble factor called smooth muscle elastogenic factor (SMEF) that appears to be produced by hypertensive but not control pulmonary artery SMCs and is capable of exerting significant effects on SMCs as well as neighboring fibroblast and endothelial cells. This factor does not demonstrate any mitogenic properties yet is capable of significantly up-regulating both the elastin- and collagen-producing capabilities of both SMCs and fibroblasts. Further, this factor is capable of inducing the elastogenic phenotype in cells not previously expressing this phenotype. Although this factor has not been identified definitively, its characteristics appear to differ from those of other mitogenic or differentiating factors produced by SMCs. Local changes in factors such as TGF-β, IGF-I, and IGF-II, agents known to stimulate elastin and collagen synthesis, may occur in response to pulmonary hypertension and act to modify SMC phenotype within the vascular wall (26, 29, 188, 189, 260, 265).

ROLE OF ENDOGENOUS VASCULAR ELASTASES AND PROTEASES IN PULMONARY HYPERTENSION Several studies have shown that activation of vascular elastase may have a primary causative role in the development of hypertension, particularly in the pulmonary artery. As described above, breaks in the internal elastic membrane are one of the first morphological events observed in pulmonary hypertension (264). Fragmentation of this elastin-rich structure implies the activation of elastases in the vessel wall.

Further, several studies have demonstrated that increases in elastase activity precede other morphological and biochemical responses, including intimal thickening, extension of smooth muscle into peripheral vessels, and increased production of elastin and collagen (142, 264, 280). Inhibition of elastase activity with the serine elastase inhibitors (SC-37698, SC-39026, and α-1-antitrypsin), during early stages of both monocrotaline- and hypoxia-induced hypertension prevents or attenuates these structural responses (142, 280), establishing that elastase activity is not only present but may in fact cause the development of pulmonary hypertension. This endogenous vascular elastase is immunologically related to, and may be a derivative of, adipsin, a serine protease first identified in adipocytes (115).

Signals for the early up-regulation of elastase activity have not been delineated fully, but recent data suggest involvement of serum factors and the cell-surface elastin-binding protein linked to tyrosine kinase activation (115).

Moreover, recent studies suggest an increase in activity of a similar elastase in accelerated neointimal formation in coronary arteries following heart transplantation (174).

As mentioned above, endothelial perturbation leads to loss of barrier function. Leak of a serum factor into the subendothelial space and vascular media could thus induce SMC elastase production or activation. The consequences of increased SMC elastase activity include breakdown of growth-inhibitory basement membranes (e.g. perlecan), subsequent activation of transcription factors (e.g. Oct-1; 274), proteolytic release and activation of growth factors stored in the matrix in an inactive form [e.g. bFGF, TGF-β], stimulation of elastin synthesis by released elastin peptides, and elastin peptide–stimulated chemotaxis (60). SMC thus activated in the vascular media would be prone to excessive proliferation when stimulated by other growth factors released by the endothelial cell or the SMC itself (Figure 7). The net effect of these processes is intimal and/or medial thickening and altered metabolism of SMCs, ultimately leading to luminal narrowing and pulmonary hypertension. Thus early increases in elastase activity, at least in smaller vessels, may have a causative role in the initiation of structural changes associated with pulmonary hypertension, and as a corollary, very early treatment with elastase inhibitors may be useful in repressing the development of hypertension.

HETEROGENEITY OF SMC RESPONSES IN PULMONARY HYPERTENSION As discussed above, there is increasing evidence that the pulmonary artery is composed of several phenotypically distinct SMC subpopulations. The possibility that these cell populations exhibit unique responses to stress was established by evaluating proliferation and matrix protein production in vivo in response to chronic hypoxia (51, 252, 278). The clusters of highly differentiated SMCs (meta-vinculin-positive cells) in the outer pulmonary arterial media of neonatal calves never expressed collagen or elastin mRNA under either normotensive or hypertensive conditions. Meta-vinculin-negative cells (in the areas between these clusters) (see Figure 4) expressed increased levels of collagen and elastin mRNA in the hypertensive state. Further, marked increases in cell proliferation occurred only in the meta-vinculin negative cells (278).

These observations suggest that not all cells in the vascular wall respond to a hypertensive stimulus in the same way. Each SMC subpopulation identified may respond differently to important stimuli (e.g. hypoxia, mechanical stress, and blood-borne or locally produced growth factors). This heterogeneity in response to various trigger factors could reflect variable responses of SMCs in various states of differentiation. Alternatively, it could provide further evidence for the existence of distinct cell populations, with different lineages, each capable of exhibiting unique responses to stress. Some SMCs may be

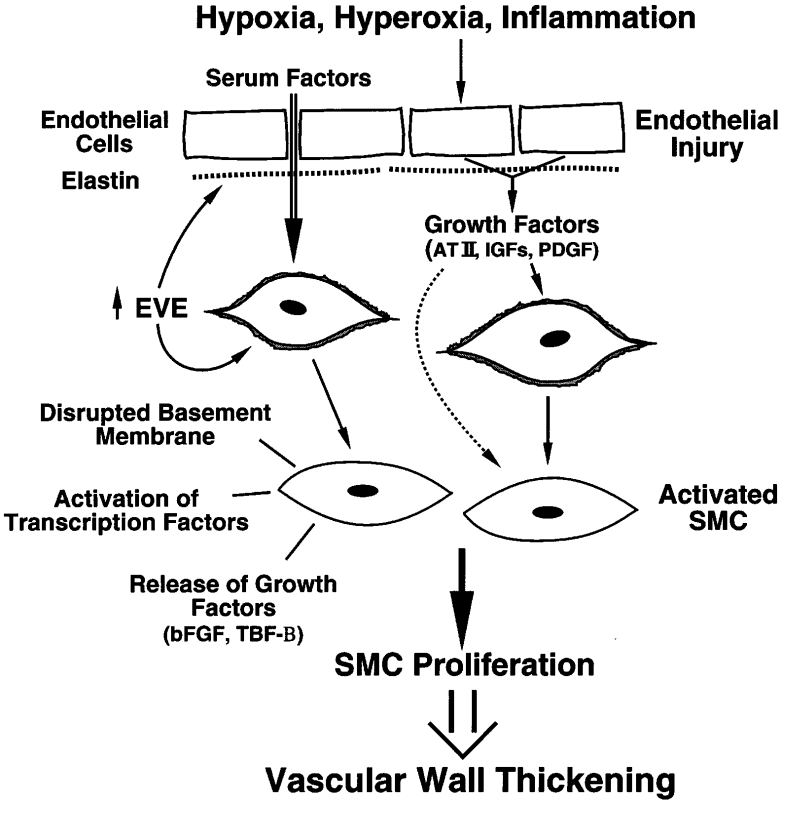


Figure 7 Schematic drawing illustrating that injurious stimuli can cause endothelial injury, leading to increased permeation of serum factors into the subendothelial space or vascular media. Concomitantly, growth-promoting factors may be released by the perturbed endothelial cell. Serum factors appear to activate endogenous vascular elastase (EVE), which may not only degrade elastin (producing elastin peptides that can stimulate SMC migration) but may also disrupt the growth-restrictive basement membrane of the mature, quiescent SMCs. This disruption leads to activation of transcription factors (e.g. Oct-1) and release of growth factors from the basement membrane. These activated SMCs may exhibit heightened growth potential to locally present growth factors. Excessive SMC proliferation occurs, leading to vascular wall thickening.

responsible for initiating early responses to trigger factors, while others may participate in the amplification of these signals. Thus it will be important to know which cells are secreting which cytokines, and which cells are expressing receptors that recognize the cytokines. Understanding how these phenotypes are modulated by environmental and developmental triggers will be important in understanding vascular remodeling in disease.

Adventitial Fibroblast Changes in Pulmonary Hypertension

The earliest and most dramatic structural changes in experimental models of pulmonary hypertension are found in the adventitial compartment. The resident fibroblasts proliferate, hypertrophy, and increase production of matrix proteins in response to locally expressed mitogens, hypoxia, and mechanical stress. What sets these cells apart from neighboring SMCs is their ability to grow directly in response to hypoxia in the absence of exogenous priming factors as well as their augmented responsiveness to mitogens and mechanical stress. Differences in signal transduction pathways and autocrine growth factor expression probably contribute to this enhanced growth and synthetic capacity as well. The existence of fewer local inhibitory mechanisms (i.e. heparan sulfates, prostacyclin, nitric oxide) in the adventitia also may be important. Finally, similar to SMCs in the medial layer, heterogeneity of the pulmonary arterial fibroblast phenotype appears to exist and likely influences susceptibility and pattern of response to chronic hypoxia.

CHANGES IN FIBROBLAST PHENOTYPE DURING THE DEVELOPMENT OF PULMONARY HYPERTENSION The response of the pulmonary vascular bed to stimuli that lead to the development of pulmonary hypertension (e.g. hypoxia and hyperoxia) differs along its longitudinal axis. While the structural remodeling process that occurs in the large conducting pulmonary arteries appears predominately in the vascular media, and the pericyte and intermediate cells play a major role in the remodeling of the pulmonary microcirculation, the structural changes in the resistance-sized muscular pulmonary arteries are most prominent in the adventitia (207, 250). Indeed, dramatic thickening of the adventitia in small muscular pulmonary arteries, including both increased cellularity and matrix protein deposition, has been observed in newborns dying from persistent pulmonary hypertension (161).

Animal models of pulmonary hypertension support findings in human pathologic specimens. In a neonatal calf model of chronic hypoxic pulmonary hypertension, for instance, the most dramatic structural changes are in the adventitial layer of small pulmonary arteries (53, 250). The structural changes observed result from rapid and dramatic changes in the proliferative and matrix protein–synthesizing phenotypes of the adventitial fibroblast. Increases in proliferation

have been demonstrated by increased BrdU uptake and by morphometric studies demonstrating that the increase in DNA synthesis results in an increase in adventitial fibroblast number (14, 53, 177).

In the rat model of hypoxic pulmonary hypertension, the adventitial fibroblast has an earlier and greater proliferative response than either the endothelial cell or SMCs (150). In hyperoxic models of pulmonary hypertension, again the adventitial fibroblast demonstrates the earliest and most dramatic increase in proliferation as measured by $^{3}[H]^{-}$ thymidine uptake (103, 104). The increase in proliferation in the hyperoxic model occurs prior to any increase in pulmonary artery pressure, suggesting that in vivo the adventitial fibroblast may be responding directly to oxygen concentration and not to later changes in flow and pressure.

In addition to an increase in the number of fibroblasts observed in the adventitia of hypoxic pulmonary arteries, marked increases in matrix protein deposition have been noted. Particularly striking have been the increases in collagen and elastin described in human and animal studies of pulmonary hypertension. Although the principal matrix component secreted by the adult fibroblast is collagen, in the normal neonatal period, very little type I collagen is produced or accumulates in the vascular wall and little to no type I collagen is produced by the fetal adventitial fibroblast (53, 155). Recent studies have demonstrated a dramatic increase in mRNA expression for type I collagen in neonatal adventitial fibroblasts during the development of hypoxic pulmonary hypertension (53). The appearance of type I collagen in the neonatal fibroblast is particularly interesting because it appears to be the result of the premature induction of type I collagen synthesis. Therefore, hypoxia-induced pulmonary hypertension seems to significantly change the ability of the neonatal adventitial fibroblast to produce type I collagen.

Significant increases in tropoelastin mRNA levels and elastin protein deposition have also been documented in the adventitia of small pulmonary arteries from chronically hypoxic calves (53, 148). This is particularly important because in the mature pulmonary artery, elastin production is normally limited to the SMCs. However, the adventitial fibroblast expresses tropoelastin mRNA during prenatal lung vascular growth and development, which then disappears during normal postnatal development. Perinatal lung injury may interfere with this normal developmental process and cause the fibroblast to re-express fetal-like proteins and genes. Indeed, both immunohistochemical and in situ hybridization studies demonstrate that the adventitial fibroblast produces elastin under chronic hypoxia (53).

During normal pulmonary vascular development, fibronectin mRNA is expressed in high levels in the fetal adventitia of small resistance-sized vessels. Expression of fibronectin mRNA by the fibroblast gradually decreases postnatally. However, in the setting of severe neonatal pulmonary hypertension

induced by hypoxia, high fetal-like levels of fibronectin mRNA continue to be expressed in the adventitia. Thus hypoxia induces various but specific changes in matrix protein expression by the fibroblast. There is a fetal-like persistence of fibronectin mRNA expression, a re-expression of tropoelastin mRNA expression, and an induction of type I collagen mRNA expression (53).

In addition to changes in the proliferative and structural protein-producing phenotypes, the pulmonary vascular fibroblast appears to undergo changes in cytoskeletal and contractile protein expression, which may significantly alter fibroblast function (217). Sappino et al (217) suggested that during normal vascular growth and development many mesenchymal cells are capable of expressing SMC markers. They speculated that the population of mesenchymal cells destined to become SMCs persist in their expression of SM contractile proteins (in particular α-SM actin), whereas this protein expression is lost in the adventitial fibroblast. There is increased expression (or perhaps re-expression) of α-SM actin by fibroblasts (myofibroblasts) in the alveolar walls and vessels in humans with pulmonary hypertension (107). In addition, cells in the adventitia of calves with severe pulmonary hypertension also express α-SM actin, suggesting conversion to a myofibrolast-like phenotype or simply re-expression of fetal protein (248). This conversion to a contractile-like cell could have dramatic functional effects on the ability of the pulmonary vasculature to respond to vasoconstrictor and dilator stimuli.

POTENTIAL MECHANISMS CONTRIBUTING TO UNIQUE CHANGES IN FIBROBLAST PHENOTYPE IN PULMONARY HYPERTENSION Fibroblasts exist as a heterogeneous cell population with functions and activity that change during development and with location within the body (105, 220). They even exist as different subsets within the same specific tissue (105). Fibroblasts also have an inherent phenotypic instability and are able to rapidly change their proliferative and secretory capabilities in response to physiologic or pathophysiologic stimuli. For instance, fibroblasts isolated from human lungs with interstitial fibrotic disease can be separated into several subpopulations that grow at different rates and react differently to mitogenic stimuli (105).

Fetal fibroblasts are especially notable for their phenotypic instability and almost malignant growth characteristics. These cells produce a PDGF-like competence factor and have a proliferative response to TGF-β that differs from adult cells (34, 92). They also synthesize different isoforms of matrix molecules from their adult counterparts and form colonies on semi-soft media, a characteristic often associated with neoplastic cells (144). In addition, fibroblasts are able to transform into myofibroblast-like cells with contractile capabilities (68). This inherent phenotypic flexibility allows them to respond quickly and

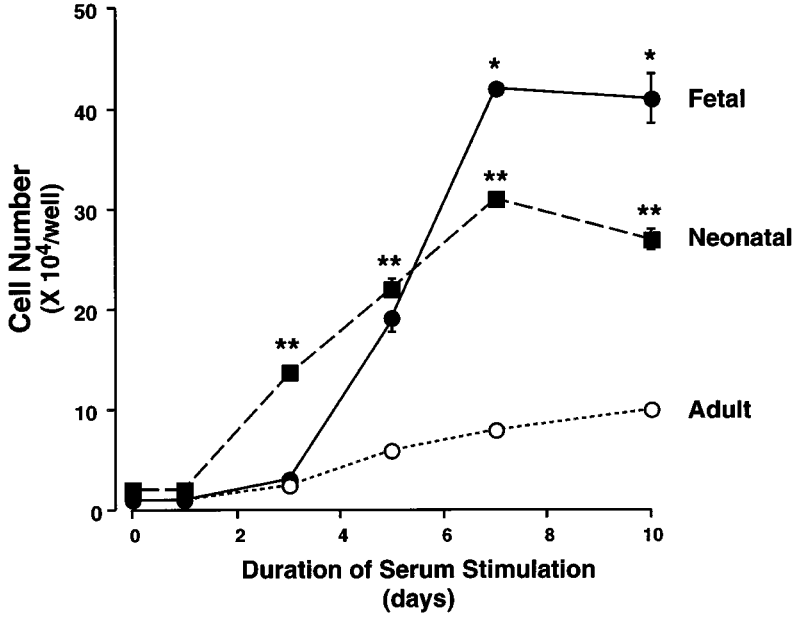


Figure 8 Serum-stimulated fetal and neonatal pulmonary artery adventitial fibroblasts grow faster and reach higher plateau densities than do adult cells. Values are means ± SE. Cells were seeded at low density (0.5×10^4 cells/cm^2) and grown in MEM/10% serum.

exuberantly to changes in surrounding conditions. However, this same inherent instability and population heterogeneity makes it difficult to study the unique responses of the fibroblast under cell culture conditions.

Proliferation of fibroblasts is enhanced in neonatal compared with adult forms of pulmonary hypertension. Recent studies have demonstrated striking developmental differences in the growth of pulmonary artery adventitial fibroblasts that appears at least partially dependent on developmental differences in the PKC signaling pathway (40). First, under serum-stimulated conditions, fetal and neonatal fibroblasts had increased DNA synthesis, grew faster, and reached higher plateau densities than did adult cells (Figure 8). The earlier during gestation that fetal fibroblasts were harvested, the more rapid the observed growth (Figure 9*a*). Serum-deprived fetal fibroblasts had increased DNA synthesis in response to a panel of potentially relevant mitogens (PMA, IGF-I, PDGF, and PMA plus IGF-I) compared with adult cells. Neonatal fibroblasts had a greater response to the PKC agonist PMA, alone and in combination with IGF-I, than did adult cells.

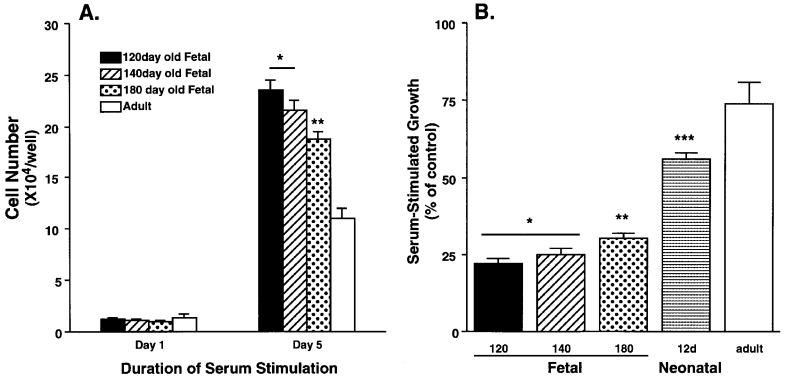


Figure 9 (*A*) Growth of fetal pulmonary artery adventitial fibroblasts depends on time of harvest during the fetal period. Fetal (120-, 140-, and 180-day-old) and adult pulmonary artery adventitial fibroblasts were seeded at 0.5×10^4 cells/cm^2 and grown in MEM-10% serum. Medium was supplemented but not replaced on day 4. *: $P < 0.05$ compared with 180-day-old fetal and adult values. **: $P < 0.05$ compared with adult. (*B*) Fetal and neonatal pulmonary artery adventitial fibroblasts are more sensitive than adult cells to the antiproliferative effects of the protein kinase C (PKC) inhibitor dihydrosphingosine (DS). On day 1, DS (3 mM) or vehicle (EtOH and 2.5 mM bovine serum albumin) was added. Cells were counted on day 1 and day 5. Similar results were obtained if DS was re-added on day 3. *: $P < 0.05$ compared with 180-day-old fetal, neonatal, and adult. **: $P < 0.05$ compared with adult and neonatal. ***: $P < 0.05$ compared with adult. Similar results were obtained with different cell populations in a second independent experiment.

Finally, using three different antagonist strategies and direct measurements of PKC catalytic activity, we demonstrated that the observed developmental differences in serum-stimulated growth were dependent on the PKC signal transduction pathway (Figure 9*b*). Thus enhanced growth of fetal and neonatal cells may contribute to the dramatic adventitial thickening observed in fetal and neonatal forms of pulmonary hypertension.

Few studies have investigated potential mechanisms contributing to the changes noted in pulmonary artery adventitial fibroblasts that occur during the development of hypoxic pulmonary hypertension. Recent studies, however, show that fibroblasts respond to lowered oxygen tension by increasing thymidine incorporation and accomplishing cell division in the absence of exogenous primiting as is required in SMCs, and that this hypoxia-induced cell proliferation depends on PKC (48, 55, 255). In addition, fibroblasts grown under conditions of hypoxia demonstrate an augmented response to mitogens, including IGF-I, IGF-II, and PDGF, in comparison with fibroblasts grown under 21% oxygen. In terms of matrix protein synthesis, fibroblasts cultured under hypoxic conditions do not demonstrate the decrease in tropoelastin synthesis seen with pulmonary

artery SMCs, and collagen synthesis increases in response to hypoxia (56). These experiments indicate a unique ability of the fibroblast to respond directly to hypoxia.

Hypoxia-induced changes in proliferation and matrix synthesis may result from local changes in growth factor production by the fibroblast. Significant increases in IGF-I and IGF-II mRNA expression are observed in vivo in the adventitial fibroblasts of neonatal calves with hypoxic pulmonary hypertension (265). IGF mRNA expression correlates directly with the observed increases in fibroblast proliferation and collagen synthesis. In vitro studies demonstrated that IGF-I and IGF-II stimulate both proliferation and matrix protein synthesis in neonatal pulmonary artery SMCs and fibroblasts. In addition, TGF-β, an established stimulus for collagen synthesis, increases under hypoxic conditions in dermal fibroblasts (58). Further, the stimulatory effect of hypoxia on procollagen mRNA levels was diminished by antibodies to TGF-β. Thus at least some hypoxia-mediated changes in fibroblast phenotype are the result of changes in growth factor production by the fibroblast.

CELLULAR MECHANISMS OF VASCULAR REMODELING IN PRIMARY PULMONARY HYPERTENSION

Studies of PPH in humans have shown two fundamental structural changes in the pulmonary vasculature: the abnormal presence of SM-like cells in normally nonmuscular compartments of the vessel and the abnormal synthesis and deposition of connective tissue proteins within different layers of the vessel wall. The muscular arteries and arterioles are the primary sites of these structural changes in patients with pulmonary hypertension. Medial SMCs extend peripherally into partially muscularized or nonmuscularized arterioles; and medial connective tissue, in particular elastin and collagen, increases (88, 273). A hyperplastic neointima develops, composed of SMCs and ECM proteins, which may partially or completely occlude the vascular lumen. Complex histologic abnormalities, such as plexiform lesions, may be observed. Interestingly, studies demonstrate marked heterogeneity in the vascular changes with some normal vessels decreased even in patients with severe and long-standing PPH. Large vessels in the adult develop a thick, fibrous neointima composed of SM-like cells, macrophages, and ECM (27). In many ways, the neointimal changes in large vessels of the hypertensive lung resemble typical atherosclerotic changes of the systemic vasculature.

Little is known about the regulation of vascular remodeling in humans with PPH. Unfortunately, animal models of pulmonary hypertension have been of limited benefit in elucidating underlying disease mechanisms in the human with PPH because remodeling in animals, in response to hypoxia, hyperoxia, or

monocrotaline, is confined largely to the media and adventitia and a neointima does not form in the elastic pulmonary arteries.. Furthermore, models involving neonatal or young animals may not apply to the disease process in adults because remodeling in younger animals must be interpreted against a context of normal vascular growth and development.

Significant advances in elucidating the etiology of the pulmonary vascular remodeling observed in PPH have been made possible because of the availability of freshly harvested human lung tissues removed from patients with PPH at the time of lung transplantation. Molecular biological techniques similar to those used to study animal models of pulmonary hypertension (see above) can be used on this tissue to determine cell phenotypes and to catalog active gene expression within different cell populations of the pulmonary vessels at the time the lung was removed from the patient.

Evidence for active remodeling in vascular tissue associated with late-stage PPH was obtained recently by Botney et al (27). Using immunohistochemistry and in situ hybridization, they found increased expression of elastin, collagen, fibronectin, and thrombospondin (all gene products characteristic of early vascular development) within the thickened intima of hypertensive vessels. Cellular changes as defined by expression of these ECM marker proteins were confined to the neointima in elastic pulmonary arteries but were observed in the media as well as intima of small muscular arteries (26, 27). Vessels in normal adult lungs demonstrated no evidence of cellular activation, at least as measured by the expression of these ECM genes.

The absence of a good animal model of human PPH has made it difficult to identify mediators of vascular remodeling. Several factors described earlier in this review as playing important roles in vascular development and in the vascular response to injury in animal models of pulmonary hypertension, including ACE (221), VEGF (267), endothelin (71), and NO (70), have been observed to change in PPH and undoubtedly play a role, although in most cases, a direct effect of these agents on the remodeling process has been difficult to demonstrate. Recent studies have focused on a possible role for TGF-β in the vascular remodeling of PPH because of its known stimulatory effect on collagen and elastin production (146) and its presence in various animal models of pulmonary hypertension.

Immunohistochemistry performed on lung tissue from patients with PPH with isoform-specific TGF-β antibodies demonstrated TGF-β2 and TGF-β3 in nearly all small muscular pulmonary arteries, in close proximity to areas of activated cells expressing procollagen (26). In large vessels, however, the findings were more complex. Immunohistochemistry revealed two populations of macrophages in the neointima of hypertensive lobar pulmonary arteries. Many macrophages resembled foam cells and tended to be found in clusters deeper

within the neointima. A second population of small nonfoamy macrophages, with a small rim of cytoplasm surrounding the nucleus, were observed more frequently within the luminal inner-third of the neointima. These nonfoamy macrophages were in proximity to neointimal cells expressing fibronectin and procollagen (135), as shown by immunohistochemistry combined with in situ hybridization. Immunohistochemistry with isoform-specific TGF-β antibodies identified all three isoforms of TGF-β associated with nonfoamy macrophages, but no immunoreactivity was associated with foamy macrophages (11). As might be predicted, large, foamy macrophages with extensive cytoplasmic vacuoles were not associated with matrix-expressing intimal cells.

Why neointimal formation occurs in human PPH but not in monocrotaline hypoxia or hyperoxic models of pulmonary hypertension injury is unknown, although recent studies suggest that the combination of vascular injury and changes in hemodynamics may direct the neointimal response. In contrast to systemic arteries, normotensive elastic pulmonary arteries rarely show neointimal or atherosclerotic changes, even in the presence of risk factors for systemic vascular atherosclerosis (11). One explanation for the absence of atherosclerosis-like lesions in pulmonary vessels is the low blood pressure in normal pulmonary arteries, rather than an intrinsic difference between pulmonary and systemic vessels (74).

The differences in hemodynamics between the pulmonary and systemic circulations raise the possibility that neointimal formation may occur in injured pulmonary arteries subjected to systemic hemodynamics. To investigate this possibility, Tanaka et al (260) created subclavian-pulmonary artery anastomoses in rats treated with monocrotaline or subjected to balloon endarterectomy injury. No remodeling, as assessed by increased ECM gene expression, was found in pulmonary vessels subjected to subclavian-pulmonary anastomosis alone. However, a non-neointimal pattern of remodeling following mild monocrotaline-induced injury was converted into a neointimal pattern in the presence of the anastomosis. Tropoelastin, type I procollagen and TGF-β gene expression was confined to the neointima and resembled the pattern of gene expression and immunoreactivity in human PPH. In the case of balloon endarterectomy, neointima was also observed even in the absence of anastomosis.

These results confirm previous observations that injury may be required to induce neointimal change in the pulmonary artery and suggest that elevated pressure alone is not perceived by the uninjured elastic pulmonary artery as a sufficient stimulus for neointimal formation. Hemodynamic conditions, however, can modulate the response to injury since neointimal formation is seen following monocrotaline-induced injury in the presence of systemic hemodynamics although no neointima is observed with monocrotaline-treatment in the

presence of normal pulmonary hemodynamics. If the injury is severe enough, such as after balloon endarterectomy, neointima will form even in the presence of normal pulmonary hemodynamics.

These findings raise the interesting possibility that injuries initiating pulmonary vascular remodeling in humans may be subtle and that the neointimal formation associated with severe pulmonary hypertension develops secondary to previous or concomitant hemodynamic changes.

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