Mechanisms of Structural Remodeling in Chronic Pulmonary Hypertension

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OBJECTIVES
After completing this article, readers should be able to:

1. Explain the physiologic consequences of structural remodeling in chronic pulmonary hypertension.
2. Describe the two primary components of the structural changes that occur in the pulmonary vascular wall during chronic pulmonary hypertension.
3. List the substances produced by endothelial cells that are affected by hypoxia.
4. Describe the potential consequences of changes in hemodynamic stresses on endothelium.
5. Delineate the primary changes in smooth muscle cells that occur in pulmonary hypertension.
6. Describe the signaling pathways that regulate responses of smooth muscle cells to stimuli.
7. Delineate the mechanisms by which hypoxia stimulates proliferation of fibroblasts.

Introduction
Pulmonary hypertension may arise in the neonate and young infant from a multitude of disease processes involving both the cardiac and respiratory systems. Common causes include congenital heart disease with increased pulmonary blood flow, diaphragmatic hernia with associated lung hypoplasia, bronchopulmonary dysplasia, and idiopathic persistent pulmonary hypertension of the newborn. Regardless of the cause, chronic pulmonary hypertension is associated with maladaptive changes in pulmonary vascular structure and function (remodeling). These changes prompt further rises in pulmonary artery pressure, limit blood flow, diaphragmatic hernia, altered pulmonary vasoreactivity, and dramatic vascular remodeling with increased pulmonary artery cellularity and synthesis and deposition of matrix protein. The data summarized in this review are derived largely from animal models of hypoxia-induced pulmonary hypertension.

Blood vessels in the lung undergo profound structural remodeling as chronic hypoxic pulmonary hypertension develops; changes include cellular hypertrophy, hyperplasia, and increased deposition of structural matrix proteins such as collagen and elastin in the vessel wall. The phenotype of cell populations that comprise the vessel wall (endothelial, smooth muscle, and fibroblast cells) change markedly and are responsible for the alterations in structure and function. However, the cellular and structural changes observed in pulmonary hypertension vary significantly, depending on patient age, duration and degree of hypoxic exposure, and the presence of associated abnormalities such as chronic inflammation or high pulmonary blood flow. Vascular responses to hypoxia involve complex cell-cell interactions that are mediated by the release of growth factors, cytokines, and biologic messengers and by changes in the composition of interstitial and basement membrane matrix proteins. Further, the cellular responses to local decreases in oxygen concentration are heterogeneous between and even among the cell populations that comprise the vessel wall. Dramatic differences have been observed in the proliferative, matrix-producing, and secretory phenotypes of different cells along the longitudinal axis of the vessel wall as well as among cells at a specific site. In addition, evidence demonstrates that the response of a particular gene to hypoxia in vivo is regulated differentially at the level of specific cell

ABBREVIATIONS
ACE: angiotensin-converting enzyme
COX: cyclo-oxygenase
ET: endothelin
HIF-1: hypoxia-inducible factor-1
IL-1: interleukin-1
ISMC: intimal smooth muscle cells
MAPK: mitogen-activated protein kinase
MMP: matrix metalloproteases
MSMC: medial smooth muscle cells
NO: nitric oxide
PKC: protein kinase-C
SMC: smooth muscle cells
TE: tropoelastin
VEGF: vascular endothelial growth factor

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types and regions within the tissue. Thus, up- or downregulation of specific gene expressions by unique or specialized cells within a tissue may influence the overall response of an organ or tissue to local changes in oxygen concentration. An understanding of the changes that occur in cells in the vascular wall when exposed to decreases in oxygen concentration and the mechanisms that cause them are critical for a better understanding of the pathophysiology of many vascular disorders, including neonatal and pediatric pulmonary hypertension.

Pathophysiologic Structural Remodeling of the Chronically Hypoxic Perinatal Pulmonary Vasculature

All forms of chronic pulmonary hypertension are characterized by both active vasoconstriction and structural changes in the pulmonary vascular wall that include cellular hyperplasia and increased production and deposition of extracellular matrix. For example, maintaining or exposing the newborn to hypoxic conditions alters pulmonary vascular structure and reactivity sufficiently to produce severe pulmonary hypertension. The resulting elevation of pressure may have both reversible (ie, vasoconstrictive) and fixed (ie, vasodilator-unresponsive) components. The vasoconstrictive component predominates in the early stage of pulmonary hypertension when there is relatively little change in the structure of the vessel wall (at least at the light microscopic level). Over time, though, the fixed component becomes more prominent (Fig. 1). The pulmonary arteries fail to dilate with the administration of either endothelial-dependent (ie, acetylcholine) or -independent (nitric oxide, sodium nitroprusside, isoproterenol) vasodilators or oxygen. The evolution of this relatively fixed, vasodilator-unresponsive component of pulmonary hypertension is related temporally to the development of thickened vascular media and adventitia, with dramatic increases in the deposition of structural matrix proteins such as collagen and elastin in the pulmonary artery walls (Fig. 2). Indeed, in our model of neonatal hypoxic pulmonary hypertension, as pulmonary artery pressure increases over a 14-day exposure to hypoxic conditions, it is the increased deposition of matrix protein in the vessel wall that we believe coincides most closely with the development of the fixed component of pulmonary hypertension.

The pulmonary circulation of infants in the immediate perinatal period may be especially vulnerable to the presence of even regional alveolar hypoxic conditions. Haworth et al have demonstrated the profound effects that perinatal hypoxia has on the structure of the neonatal pulmonary vascular bed (see Suggested Reading). When fetal levels of hypoxia were maintained postnataally in the large white pig, the fetal pulmonary circulation did not undergo the full dramatic remodeling that normally occurs after birth. Pulmonary arterial endothelial and smooth muscle cells from the animals that had been exposed to chronic hypoxia at birth maintained the shape, overlap, and interdigitation characteristics of fetal life, which resulted in an increased pulmonary artery medial thickness compared with normoxic controls. In the hypoxic neonatal calf model, the histologic structure of the small pulmonary arteries from calves made hypoxic for 1 and 3 days was very similar to that of the newborn just hours old. In addition, the pulmonary artery resistances and pressures in the hypoxic animals failed to regress. Thus, instead of the normal thinning of the pulmonary arterial wall and increase in vascular lumen diameter that results in a concomitant fall in pulmonary artery pressure and resistance, the small pulmonary arteries of calves remained thickened and had evidence of increased deposition of pulmonary vascular extracellular matrix after 2 weeks of exposure to hypoxia. These findings are consistent with those described in human neonates who have pulmonary hypertension in whom there is a significant element of fixed pulmonary hypertension.

Alterations in Endothelial Cells During Development of Chronic Hypoxic Pulmonary Hypertension

The endothelium forms a nonthrombogenic, semipermeable barrier between the blood stream and all extravascular tissues and fluid compartments in the body. In addition, it

![FIGURE 1. Responsiveness to vasodilators decreases during the development of pulmonary hypertension.](http://pedsinreview.aappublications.org/)
influences vascular tone, hemostasis, growth, differentiation, chemotaxis, and the response of other vascular compartments to injury. Given this important role in such a wide array of vascular functions, it is not surprising that significant adaptations occur in the structure and function of the endothelial cell during the development of hypoxic pulmonary hypertension.

HISTOLOGY/MORPHOLOGY
Several studies have characterized the histologic changes that occur in the endothelial cells of both large and small vessels in response to chronic hypoxic exposure. Hypoxia increases intimal thickness by causing hypertrophy and hyperplasia in both the endothelial and subendothelial layers. Endothelial cell hypertrophy is associated with increased numbers and size of cell organelles, including ribosomes, rough endoplasmic reticulum, and golgi apparatus. Increases in DNA synthesis and cell number are demonstrated by the approximately three-fold increase in $^3$H-thymidine incorporation observed early in the course of hypoxic exposure. Diffuse subendothelial edema also occurs. The number of pinocytic vesicles increases, as do the intracellular gaps between endothelial cells. There is often focal disruption and lysis of the endothelial cell basement membrane that creates a patchy appearance of microfibrilar 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. Under normal conditions, it appears that endothelial cell production of elastin is suppressed sometime in late fetal or early neonatal life. It is very likely that the endothelial cell re-expresses tropoelastin mRNA in response to injury. In addition, smooth muscle cells (SMC) are recruited into this enlarging subendothelial space, which could contribute to the accumulation of protein in this space.

CHANGES IN GROWTH FACTOR AND VASOACTIVE SUBSTANCE PRODUCTION
Hypoxia disturbs endothelial function by altering the regulation of vascular tone, increasing permeability, reducing the antithrombotic activities of the endothelium, and promoting release of cytokines and growth factors. The relationship between the vasoconstrictor response to acute hypoxia and the structural remodeling characteristic of chronic hypoxia is not understood. The onset of hypoxia that is associated with acute changes in vasoreactivity and sustained hypoxia influences the
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Chronic Pulmonary Hypertension

activity of certain transcription factors for hypoxia-sensitive genes, several of which promote cell growth. Table 1 lists many of the growth- and tone-modulating substances synthesized by the vascular endothelium whose production is known or believed to be influenced by hypoxia.

The endothelium helps to regulate the tone and growth of the underlying vascular SMC through continuous production of various vasoactive mediators. Chronic exposure to hypoxia reduces the production of both prostacyclin and nitric oxide (NO). Hypoxia also can inhibit uptake of the NO precursor L-arginine by pulmonary arterial endothelial cells within 4 hours and may suppress expression of NO synthase and activity. Although inhibition of prostacyclin and NO is not believed to cause hypoxic vasoconstriction, both substances are antiproliferative, with NO acting via cGMP and prostacyclin through a cAMP-mediated effect. Thus, a reduction in their production could contribute to proliferation of SMC or fibroblasts.

Hypoxia may modulate the inhibitory effect of endothelial cells on vascular cell proliferation by other mechanisms. NO can induce reversible inactivation of the protein kinase-C (PKC) pathway, an intracellular signaling pathway associated with cell proliferation. Normal endothelial cells also secrete heparan sulfate, which directly inhibits growth of SMC by a posttranslational PKC-dependent mechanism. Hypoxia has been shown to inhibit release of heparan sulfate from endothelial cells, thereby countering the normal endothelial mechanisms that act to restrain proliferation of SMC.

The contribution of vascular endothelial growth factor (VEGF) to the hypoxic response is not yet clear, although VEGF, VEGF mRNA, and the transcripts for its receptors are upregulated in chronic hypoxia. VEGF may be important in regulating release of NO. It also increases vessel permeability and can stimulate prostacyclin synthase. Although VEGF and its receptors have been located on the pulmonary vasculature, most of the mechanistic studies have not been carried out on pulmonary vascular endothelial cells.

Vasoconstrictor substances produced by the endothelium include endothelin, thromboxane A2, angiotensin II, prostaglandin H2, leukotrienes, platelet activating factor, and superoxide anion. Hypoxia is associated with an increase in the plasma level of endothelin (ET)-1 and increased gene transcription for ET-1 and for the ETA receptor on the SMC. It also increases the synthesis and release of angiotensin-converting enzyme (ACE) and stimulates the uptake of serotonin in pulmonary arterial endothelial cells. Several powerful vasoconstrictors, such as ET and angiotensin, also promote SMC growth, and some can enhance growth further by stimulating the production of other growth factors. Thromboxane A2 and angiotensin II, for example, stimulate and increase the expression of basic fibroblast growth factor and insulin-like growth factor-I, respectively. ACE activity is reduced in chronic hypoxic rat whole lungs, but ACE protein and gene expression is upregulated in newly muscularized peripheral pulmonary arteries. ACE inhibition with captopril attenuates muscularization of pulmonary vessels and the severity of the pulmonary hypertensive response to hypoxia. Also, stimulation of complementary signal transduction pathways by different stimuli can have pronounced synergistic effects. PKC and mitogen-activated protein kinase (MAPK) appear to play central roles in the interaction of mechanical, hypoxic, and growth factor-induced responses.

Hypoxia also increases the expression of several cytokines and growth factor genes in vitro and in vivo. The expression of platelet-derived growth factor-B is increased in the hypertensive lungs of hypoxic rats. Interleukin-1α (IL-1α)-dependent upregulation of ICAM-1 is seen in pulmonary microvessels of intact hypoxic mice, and hypoxia-induced increases in IL-8 were mimicked by increases in IP-10 (murine homologue) in hypoxic mice. The mechanism by which hypoxia upregulates responsive genes is not clear. However, the transcription factor known as hypoxia-inducible factor-1 (HIF-1) is induced in all cell types tested, and the hypoxic response elements of target genes such as VEGF, erythropoietin, and several glycolytic enzymes are recognized and regulated by this transcription factor. HIF-1 is redox-sensitive. Activation of several hypoxia-related genes appears to involve a heme protein, possibly acting as an oxygen sensor. These include the genes that encode for ET-1, VEGF, tyrosine hydroxylase, and erythropoietin.

Thus, by regulating vascular tone, permeability, and production of growth factor, the endothelium plays a central role in modulating the pulmonary vascular response to hypoxia and ultimately the pathologic structural remodeling that occurs during the development of chronic pulmonary hypertension. There is now an overriding need to understand how to direct and manipulate the endothelial cell signal transduction pathways that control the potentially protective, beneficial effect of certain vasoactive substances and the signaling pathways that control structural remodeling.

Response of Endothelium to Hypoxia-induced Hemodynamic Stress

Exposure to acute or chronic hypoxia elicits significant changes in the hemodynamic stresses imposed on the pulmonary circulation. It is difficult to separate the cellular responses induced by hypoxia from hemodynamic forces in vivo because they may act cooperatively or in some instances synergistically. Thus, to comprehend the remodeling associated with chronic hypoxia, the effects of hemodynamic forces, particularly sheer stresses, on the endothelium must be understood. Blood flow is an important acute modulator of vascular tone, but it also can influence the more chronic process of vascular remodeling. Many flow-induced effects result, at least in part, from the ability of the endothelium to sense and transduce hemodynamic stimuli into changes in vascular structure and function. Examples include increased microvascular permeability, lipoprotein accumulation, leukocyte adhesion...
molecule expression, mononuclear leukocyte recruitment, and vessel size changes following alterations in SMC proliferation or matrix protein synthesis.

Almost all available evidence tends to support the hypothesis that the endothelium is the site of shear stress transduction in the arterial wall. Multiple endothelial shear sensors elicit responses via interlinked signal transduction pathways. Recent work suggests shear sensing at focal adhesion plaques and at G protein-coupled receptors. However, shear stress-sensitive ion channels also have been identified. It is not surprising that shear stress drives multiple signal transduction cascades, given the spectrum of physiologic responses and the large panel of genes that shear regulates, probably via a large array of transcriptional regulators.

Endothelial cells not only transduce shear stress and elaborate a number of genes, including vasoactive and growth-promoting cytokines that induce remodeling of the arterial wall, but the response to most hemodynamic stresses begins at the level of the endothelium. It appears that cell shape, orientation, and cytoskeletal organization all are sensitive to shear stress. Although this morphologic sensitivity is well known, its regulation is poorly understood. Changes in cell shape induced by shear stress have potentially important implications for physiologic functions of the endothelium. They necessitate the partial disassembly and reassembly of the adherence junction, a cadherin-based protein complex that mediates cell-cell adhesion. Reassembly is not completed until 24 to 48 hours after shear stress is imposed. Disruption of the adherence junction compromises the endothelial permeability barrier, which may help to explain the high permeability of endothelium seen at vascular sites exposed to altered flow conditions. Changes in permeability are believed by some to be a critical first step in initiating the remodeling process.

The structural changes associated with long-term alterations in blood flow involve alterations in the tissue content of the vessel wall and its reorganization. Depending on the pathophysiologic condition, there may be increases in the number of SMC and significant changes in the content of matrix protein. Reorganization of the critical matrix protein elastin and net accumulation are important in vascular remodeling. It appears that modifications in vessel diameter elicited by hemodynamic changes are due to shear stress-induced alterations in endogenous vascular elastases as well as changes in matrix metalloproteinases (MMP), including MMP-2 and MMP-9. Thus, the endothelium seems to have profound effects on the structure and composition of the vessel wall under changing hemodynamic conditions. It is not surprising, therefore, that hypoxia and changes in blood flow may act synergistically under certain conditions to cause dramatic structural alterations in the pulmonary circulation. This has important clinical relevance in that certain conditions characterized by high pulmonary blood flow may be exacerbated by local hypoxic conditions within the lung to cause dramatic structural remodeling.

**Alteration in SMC Function and Phenotype**

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 proliferation of SMC, hypertrophy, and deposition of matrix protein. Hypoxia, mechanical stress, and blood-borne and locally produced mitogens act collectively to drive these cellular responses. They activate a cascade of intracellular signaling mechanisms, including tyrosine kinases, calcium (Ca++) MAPK, and PKC, that promote growth of SMC and synthesis of matrix protein. Synergy between different stimuli and resulting “crosstalk” between signal transduction pathways augments the extent of vascular changes. Susceptibility to these stimuli is enhanced when inhibiting mechanisms are impaired, such as endothelial barrier function, local production of heparan sulfates, and prostacyclin- and NO-induced increases in cyclic nucleotides. Intrinsic (developmental, genetic, acquired) differences in growth and matrix synthetic capacity and local and regional phenotypic heterogeneity of pulmonary artery SMC also regulate the pattern of remodeling in the tunica media in response to chronic hypoxia.

**HISTOLOGY/MORPHOLOGY**

Increases in the thickness of the medial layer of normally muscular arteries and an extension of muscle into smaller and more peripheral vessels are common to all forms of human pulmonary hypertension. Detailed characterization of the changes in SMC induced by chronic hypoxia have been examined in animal studies.

The timing and nature of changes in SMC of both the proximal and distal pulmonary vasculature differ. In the proximal pulmonary arteries of adult rats, the media thickens due to hypertrophy and hyperplasia of the individual muscle cells and increased synthesis and deposition of extracellular matrix proteins, elastin, and collagen. In the distal vasculature, the muscularization of previously nonmuscular arteries or so-called “extension” is brought about by differentiation and hypertrophy of cells (intermediate cells and pericytes) present in the wall. In addition, some investigators believe that interstitial fibroblasts are recruited locally into a cell that exhibits muscle-specific proteins and functions. Both the intermediate cell and the pericyte undergo significant changes and acquire a more smooth muscle-like appearance. 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. Because this internal lamina is not as complete as in normal muscular arteries, the endothelial cell and new muscle cells form frequent contacts. These contacts differ from those observed in normal muscular pulmonary arteries, suggesting that a close and perhaps different communication might exist between these cells in the newly muscularized vessels.

If hypoxic exposure occurs at or around the time of birth, different cellular responses are noted than if hypoxia occurs later in life. The
Peripheral pulmonary arteries of newborns who die in the first 36 hours after a hypoxic event demonstrated an extremely thick-walled fetal-like structure. The SMC of all animals exposed to hypoxia during the first week of life demonstrate an increased concentration of myofilaments. In the large arteries, the adluminal SMC (on the outside of the vessel) exhibit a greater increase in myofilaments than do the abluminal cells (on the inside of the cell). This very rapid and dramatic response in neonates requires a much longer period of exposure to elicit in older animals.

**EFFECT ON VASCULAR MATRIX PROTEIN PRODUCTION**

Marked increases in deposition of matrix protein are noted in the hypoxic neonatal pulmonary vascular wall. The deposition of matrix proteins in blood vessel walls is crucial for normal blood vessel structure and function, and an increase could influence dramatically the vascular response to changes in hemodynamics and other stimuli. Because of its importance, the complex pattern of structural matrix protein expression is tightly regulated throughout development. For example, tropoelastin mRNA is highly expressed in the blood vessel wall primarily during late fetal and early neonatal life, but it decreases rapidly with increased maturity until virtually no expression is detected in adult vessels. Further, regulation of matrix protein expression during development may differ even within a tissue such as the pulmonary vascular system. Such differences may vary along the longitudinal axis of the pulmonary artery and, as will be discussed, vary between specific subpopulations of SMC within the same pulmonary artery wall.

We investigated the hypothesis that maintaining high pulmonary artery pressure, as occurs in the hypoxic neonatal calf model of pulmonary hypertension, might change the normal postnatal pattern of extracellular matrix protein expression in newborn pulmonary arteries. Such changes ultimately would result in excessive production of new protein that would contribute to pathologic pulmonary arterial remodeling and subsequent pulmonary hypertension that is unresponsive to conventional therapies. To investigate this hypothesis, we examined the normal developmental expression of three extracellular matrix proteins—fibronectin, tropoelastin, and alpha-1 (I) procollagen and fibronectin—in small resistance-sized pulmonary arteries of both fetal and neonatal calves and assessed the impact of severe pulmonary hypertension induced by hypoxia on their expression. In situ hybridization was used to localize and assess expression of these matrix mRNAs. The developmental regulation of tropoelastin mRNA expression is both tissue- and species-specific. For example, tropoelastin mRNA expression and elastin deposition peak postnatally in the rat, which is born with relatively immature lungs. In contrast, tropoelastin mRNA expression in small resistance pulmonary arteries occurs late in gestation in the calf, which is born with more mature lungs. In the neonatal calf model of hypoxic pulmonary hypertension, small muscular pulmonary arteries, which had ceased tropoelastin expression in normal, normoxic animals at birth, re-expressed tropoelastin mRNA postnatally in response to hypoxia (Table 2). The strongest tropoelastin mRNA signal in small resistance pulmonary arteries localized to the outer medial and adventitial layers of pulmonary hypertensive arteries. Thus, maintaining hypoxia early in the postnatal period resulted in the abnormal re-expression of tropoelastin mRNA in pulmonary arteries that had ceased expression in utero. This expression ultimately results in increased elastin deposition in the vessel wall as it undergoes pathologic vascular remodeling.

Type I collagen is a rigid structural matrix protein that does not appear to be necessary for normal early lung development. We detected little or no alpha-1 (I) procollagen mRNA in muscular pulmonary arteries from late-gestation fetal calves or during normal postnatal life, although expression was present in large elastic arteries and in the lung interstitium (Table 2). However, during exposure to chronic hypoxia, alpha-1 (I) procollagen expression was induced rapidly in the muscular pulmonary artery. Because of the high tensile strength of collagen, the increase in alpha-1 (I) procollagen expression and subsequent protein deposition increase the ability of the pulmonary artery

### TABLE 2. Relative Signal* of Fibronectin, Tropoelastin, and Alpha-1 (I) Procollagen mRNA in Muscular Pulmonary Arteries

<table>
<thead>
<tr>
<th>AGE (DAY)</th>
<th>FIBRONECTIN</th>
<th>TROPOELASTIN</th>
<th>ALPHA-1 (I) PROCOLLAGEN</th>
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<tbody>
<tr>
<td></td>
<td>NORMOXIA</td>
<td>HYPOXIA†</td>
<td>NORMOXIA</td>
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<tr>
<td>Fetus</td>
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<td>+</td>
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<td>2</td>
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<td>8</td>
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<tr>
<td>15</td>
<td>–</td>
<td>+++</td>
<td>–</td>
</tr>
</tbody>
</table>

*Relative signal strength was estimated by comparing probe specific activity, exposure time, and grain density per area.
†Length of exposure to chronic hypoxia was age minus 1 day.

to withstand the higher vascular pressure and flow that accompany the development of hypoxic pulmonary hypertension. This same process, however, may alter the reactivity of the vessel and restrict its ability to vasodilate in the face of higher pressure and flow, as occurs when neonatal calves and other experimental animals are exposed to chronic hypoxia.

Fibronectin is a 460-kd glycoprotein dimer that helps to control cell migration, division, and differentiation. In accordance with its important role during embryologic development, we found very high fibronectin mRNA levels in late-gestation fetal calf pulmonary arteries (Table 2). Postnatally, these levels decreased and became undetectable by 15 days of life. The late fetal pattern of fibronectin expression persisted in small pulmonary arteries from chronically hypoxic animals, with the mRNA signal localized predominantly to the outer medial and adventitial areas of the pulmonary artery wall. Because fibronectin maintains cells in a more synthetic and proliferative phenotype, the persistence of such expression in the pulmonary arteries of chronically hypoxic neonatal animals that have pulmonary hypertension might maintain or induce cells in the outer media to remain highly proliferative, mobile, and capable of rapidly increasing matrix protein. These cells, therefore, would be more likely to respond rapidly to changes in pulmonary vascular pressure and blood flow with increased proliferation and structural protein synthesis and deposition.

The development of pulmonary hypertension also may alter the expression of proteolytic enzymes and other matrix proteins that have growth-regulating activities. The proteolytic activity of elastases and metalloproteinases in the vessel wall has been shown in both cell culture and whole animal studies to induce the release of mitogenic growth factors from the extracellular matrix. In addition, upregulation of the matrix glycoprotein tenascin, which has been identified in both humans and experimental animals that have pulmonary hypertension, is accompanied by the proliferation of SMC.

Interestingly, the tenascin effect on cell proliferation appears to be regulated by elastase and metalloproteinases; that is, when collagen is proteolyzed, integrin binding sites become exposed. When bound, tenascin gene transcription is induced via MAPK. It is of interest that when MMP or elastases are inhibited, hypertrophied pulmonary arteries regress in organ culture. This is associated with downregulation of expression of tenascin.

**UNIQUE RESPONSES OF SPECIFIC SMC SUBPOPULATIONS TO HYPOXIA**

The vascular media (at least of large vessels) is not composed of a single population of SMC, but rather is a mosaic of phenotypically distinct subpopulations. New investigations are examining the hypothesis that specific subsets of arterial SMC contribute selectively to the vascular response to injury. A substantial body of experimental evidence demonstrates that pathologic lesions in atherosclerosis, restenosis, and hypertension in humans (as well as the neointima in injured animal vessels) are composed primarily of cells that have nonmuscle-like characteristics. The absence or paucity of muscle-specific markers in these cells historically had been attributed to a process of “phenotypic modulation” of a differentiated medial SMC type during its proliferation and migration into the intimal space. However, the identification of cells with nonmuscle-like characteristics in the normal mature vascular media indicate that pathologic lesions in the arteries also could originate via expansion of a subset of relatively “undifferentiated” or “immature” medial cells. For example, it has been suggested that the intimal thickening seen after balloon injury in canine arteries results from selective proliferation of a subset of nonmuscle-like cells in the arterial media. Recent studies demonstrating the monoclonality of atherosclerotic plaques in human arteries also raised the possibility that unique subsets of medial cells participate in the pathologic response of the vessel wall to injury.

We found marked heterogeneity in the proliferative and matrix-producing responses of pulmonary medial SMC to the stimuli associated with hypoxic pulmonary hypertension. Using double-label immunofluorescence staining, phenotypically unique subpopulations of SMC in bovine pulmonary arteries demonstrated markedly different proliferative responses to hypoxia-induced pulmonary hypertension. At every posthypoxic time point studied, greater than 95% of the proliferation occurred within a subpopulation that had no expression of the muscle-specific marker metavinculin; whereas a population of SMC expressing metavinculin remained quiescent. These findings argue that the population of metavinculin-negative cells is functionally distinct from a metavinculin-positive population of cells. This argument is strengthened when the matrix protein expression activity of these subpopulations of SMC is assessed. In the normal bovine pulmonary artery, a subpopulation of SMC acquires the ability to express metavinculin during development. These metavinculin-expressing cells no longer express tropoelastin (TE), while non metavinculin-expressing cells continue to express TE mRNA. Indeed, even with the development of hypoxic pulmonary hypertension during which TE expression is greatly increased in other subpopulations of SMC, the subpopulation of metavinculin-positive cells does not express TE.

Initial observations in the systemic circulation of two populations of SMC that had markedly different morphologies and growth patterns (ie, one isolated from the neointima of injured rat carotid arteries and the other from the unmanipulated tunica media) supported the hypothesis that the arterial wall may be composed of different types of SMC. It also is possible that neointima originates via expansion of a small pre-existing medial population of SMC that has enhanced potential for proliferation and extracellular matrix protein synthesis rather than simply by phenotypic modulation of the overall medial population of SMC. Investigators recently demonstrated that two morphologically and functionally distinct types of SMC could be
isolated from different compartments of the vascular media at the same site in the normal rat aorta. Aortic medial cells (MSMC) were isolated from the media after removal of the intima and adventitia. Aortic intimal cells (ISMC) were isolated from the luminal side of everted rat aortas by scraping. MSMC were spindle-shaped, grew in the hill-and-valley pattern traditionally described for differentiated vascular SMC, and expressed alpha-SM-actin and SM-myosin. Conversely, ISMC displayed a polygonal or epitheloid shape, grew mainly as a monolayer, expressed alpha-SM-actin but not SM-myosin, and were negative for Factor VIII antigen. ISMC produced large amounts of a laminin- and type IV collagen-rich extracellular matrix, which had a unique and characteristic pericellular distribution. Contractile responses to ET differed significantly between the two populations. Interestingly, the ISMC isolated from the normal rat arteries exhibited characteristics very similar to those reported for neointimal cells isolated from injured carotid arteries.

We also have performed cell culture studies using pulmonary and systemic arteries from the neonate of a large mammalian species (bovine), with the goal of isolating and maintaining in culture heterogeneous arterial subpopulations of SMC that exhibited unique characteristics similar to those observed in vivo. We isolated four phenotypically unique populations, each exhibiting distinct morphological and biochemical characteristics. The isolated cell populations could be split broadly into two major categories that exhibited either smooth muscle characteristics or “nonmuscle-like” features. Interestingly, the cell subpopulations isolated from the bovine species demonstrated many morphological, biochemical, and functional characteristics similar to cell subpopulations derived from the normal rat arterial media. As was the case with the rat cells, the observed morphological and biochemical differences were maintained by the distinct cell populations over multiple passages in culture. None of the populations modulated into another phenotype, supporting the possibility that they represented distinct cell lineages.

Cells exhibiting unique morphological and biochemical characteristics also differed significantly with respect to their proliferative responses to growth-promoting stimuli. In general, the “nonmuscle-like” cell subpopulations exhibited markedly enhanced growth capabilities under serum-stimulated conditions compared with “differentiated” populations of SMC. Moreover, nonmuscle-like cells exhibited the capacity to grow in plasma-based media; the differentiated SMC remained quiescent. We also found specific subsets of cells among the nonmuscle-like cell populations that proliferated autonomously, similar to embryonic aortic cells.

Because in vivo studies have demonstrated selective proliferative responses of arterial SMC to hypoxia-induced pulmonary hypertension, we examined the possibility that distinct cell subpopulations isolated from the normal arterial media also would exhibit different responses to hypoxia in vitro. Indeed, only specific cell populations proliferated in response to hypoxia. In general, the nonmuscle-like cells compared with differentiated SMC, whose growth was inhibited under hypoxic conditions.

**SIGNSIGNAL PATHWAYS IN SMC SUBPOPULATIONS**

The remarkable differences in proliferative and matrix protein synthetic responses to similar stimuli between different medial subpopulations of SMC suggest that the membrane receptor or intracellular signaling pathways controlling these responses may differ substantially. The contribution of the receptor tyrosine kinase and the G protein-coupled pathways to growth vary between vascular SMC isolated from different species. For example, cells isolated from the subendothelial space, which exhibit markedly enhanced growth potential, use a pertussis toxin-sensitive G protein-coupled pathway that does not appear to contribute to growth in the other cell types in the bovine arterial wall. Additional studies using stable transfection have shown that overexpression of specific G proteins in vascular cells significantly changes the functional responses of the cell to external stimuli.

Even in the absence of obvious receptor or membrane differences, cells could exhibit different responses to similar stimuli based on the presence of or differential activation of downstream effector pathways. Recently it was demonstrated that the MAPK pathway can mediate either growth inhibition or proliferation in different human vascular types of SMC, depending on the availability of specific downstream targets. Some human vascular SMC expressed the inducible form of cyclooxygenase (COX-2), and other SMC did not. In those cells that expressed COX-2, activation of MAPK served as a negative regulator of proliferation, in contrast to SMC that lacked COX-2, in which MAPK activation led to proliferation. In cells expressing COX-2, platelet-derived growth factor-induced MAPK activation led to cytosolic phospholipase A2, activation, prostaglandin E2 release, and subsequent activation of the cAMP-dependent protein kinase (PKA), which strongly inhibits SMC proliferation. Thus, the biologic outcome in response to similar stimuli, at least in SMC mediated by MAPK, is highly dependent on downstream enzymes expressed by the cell, which is not unexpected. It is interesting that the only subpopulations of SMC that appear to exhibit enhanced proliferative responses to hypoxia, in the absence of exogenous mitogens or serum, are those that demonstrate constitutive activation of extracellular signal-regulated kinase (Erk) 1 and 2.

Another intracellular signaling pathway that exerts a wide variety of effects on different cell processes is the PKC pathway. Numerous isozymes of PKC are regulated both developmentally and in a cell-specific manner. Thus, specific PKC isozymes and their susceptibility to activation could confer unique properties to different cell types. Because PKC activation can contribute to a general pattern of overall enhanced growth capacity, we tested whether the cell subpopulations susceptible to hypoxic growth stimulation would
exhibit different patterns of PKC isozyme expression than those whose growth was inhibited by hypoxia. Further, because the alpha-isozyme of PKC (a calcium-dependent isoformal) is an important determinant of hypoxic growth capacity, we compared the level of expression of PKC-alpha isoform in the two medial SMC subtypes and found that nonmuscle-like cells had increased levels of immunodetectable PKC-alpha compared with the nonproliferative differentiated SMC. This pattern of isozyme expression was paralleled by increased whole cellular PKC catalytic activity in the hypoxia-sensitive compared with hypoxia-insensitive cells. Thus, distinct arterial cell subpopulations, similar to those observed in vivo, were isolated and maintained in culture and demonstrated unique differences in the signaling mechanisms that appear to contribute to their unique growth responses.

**UNIQUE RESPONSES OF ADVENTITIAL FIBROBLASTS**

The possibility that hypoxia acts directly or in unique ways on the adventitial fibroblast in the setting of chronic hypoxic pulmonary hypertension is raised by previous observations that reduced oxygen tension (anoxia/hypoxia) results in profound changes in fibroblast physiology and metabolism. Cellular anoxia is a biochemical state that is distinct from hypoxia yet still represents a normal physiologic condition during wound healing. Because a response that differs substantially from that seen with hypoxia is induced in anoxic fibroblasts, these cells must possess a unique ability to activate a different, possibly overlapping set of genes to cope with these different environmental conditions. The activity of several different transcription factors is influenced by low oxygen tensions. In cells that are stressed by oxygen deprivation, NF-K B activity increases as a result of phosphorylation and subsequent degradation of I K B-alpha. In other cells, low oxygen tensions induce the transcription of multiple members of the basic/leucine zipper domain superfamily, result in nuclear accumulation of p53, or induce the activity of HIF-1. It has been demonstrated that one DNA binding activity induced in hypoxic, anoxic, and cobalt-treated fibroblasts recognizes secondary anoxia-responsive elements and has electrophoretic mobility similar to that of HIF-1. Identification of a mammalian anoxic response element may prove useful in gene therapy regimens for targeting expression to physiologic situations of functional anaerobiosis, such as during wound healing. Interestingly, deregulation of genes normally expressed during anoxia is common in cancer cells regardless of their state of oxygenation. Thus, understanding the molecular basis of the mammalian anoxic regulatory pathway in fibroblasts and how similar genes become constitutively activated in malignancy may lead to unique approaches to therapeutic intervention for vascular disease complicated by hypoxia.

Recent observations in systemic models of vascular injury suggest that early activation and subsequent phenotypic modulation of the fibroblast is an important and perhaps ubiquitous response in the vascular remodeling that follows stress or injury. For example, a sequence of events occurring in adventitial fibroblasts of the coronary vasculature following balloon catheter-induced injury is similar to that seen in the skin wound healing process. Cell proliferation is an early phenomenon that may involve the entire adventitia of the blood vessel. The proliferation observed in the adventitia occurs earlier and is of greater magnitude than is seen in the coronary media. Subsequently, fibroblasts in the coronary artery adventitia differentiate into myofibroblast-like cells, with alpha-SM actin appearing in adventitial cells as early as 3 days and reaching a maximum at 14 days. These changes in proliferation and contractile protein expression in adventitial cells are accompanied by the induction of procollagen alpha-1 mRNA and subsequent protein accumulation in the adventitial compartment. Additionally, recent studies suggest that these activated fibroblasts (myofibroblasts) may migrate through the vessel wall and be at least partially responsible for the intimal thickening that ultimately characterizes the coronary vasculature following balloon injury.

**Adventitial fibroblasts also are essential in the remodeling changes of venous grafts after they are placed in the arterial system. The appearance of myofibroblasts and ultimately a collagenous scar may contribute to the failure of aorto-coronary saphenous vein grafts to undergo compensatory dilatation when atherosclerotic lesions begin to compromise the lumen. Additionally, in hypoxic models of lung injury, fibroblasts are activated, migrate, and acquire smooth muscle-like characteristics in the small pulmonary arteries. Fibroblasts in the pulmonary artery adventitial layer,**
much like SMC in the outer media, also demonstrate dramatic increases in tropoelastin, collagen, and fibronectin expression during the development of chronic hypoxic pulmonary hypertension (Table 2). Fibroblasts in these settings are suspected of being the “source” of cells in newly muscularized vessels. Thus, observations in both the pulmonary and systemic circulations suggest ubiquitous involvement of adventitial cells in the vascular repair process.

FIBROBLAST INTERACTIONS WITH OTHER CELL TYPES
A large body of experimental evidence demonstrates that fibroblasts may exert significant phenotypic effects on other cell types, raising the possibility that they contribute to the vascular remodeling process in dynamic ways that are in addition to direct changes in their phenotype. The existence of dynamic and reciprocal relationships between fibroblasts and other cell types is well documented, especially in the developmental biology literature.

The biochemical identity of signal molecules that mediate mesenchymal-epithelial interactions has been investigated intensively, and it has been established that matrix proteins such as collagen, fibronectin, and proteoglycans play a prominent role. There is continuous feedback of information between cell and matrix. Specific matrix molecules interact with their receptors at the cell surface in a diverse array of cell behaviors. Fibroblasts also produce a number of soluble factors that function as paracrine regulators of proliferation, migration, and biosynthetic activity among neighboring cells (endothelial cells, SMC, epithelial cells). The biologic activity of the soluble factors and the nature of the extracellular matrix in contact with the cells are mutually interdependent, with soluble factors (eg, transforming growth factor beta, epithelial growth factor, insulin-like growth factor) exerting effects on matrix biosynthesis and the response of cells to these factors being modulated by the nature of the matrix. Thus, fibroblasts may have significant effects on neighboring vascular wall cells and could contribute in unique ways to the vascular remodeling process.

Recent experiments have demonstrated the importance of fibroblast communication in vascular injury models. For example, application of the inflammatory cytokine IL 1-beta to the adventitia induces coronary vasospasm and neointimal formation even without endoluminal manipulations. These findings are relevant to clinical settings because the accumulation of mast cells and an inflammatory reaction occur in patients who have coronary vasospasm and fatal unstable coronary syndromes, respectively. Similarly, molecules that inhibit cellular proliferation when applied to the adventitia decrease the development of intimal thickening in response to luminal injury. Thus, a substantial body of in vivo and in vitro evidence suggests a dynamic reciprocity in the interaction between adventitial fibroblasts and other vascular walls similar to the dynamic interactions between mesenchymal cells and epithelium during development.

MECHANISMS OF HYPOXIA-STIMULATED FIBROBLAST PROLIFERATION
As suggested previously, PKC is one important intracellular signalling pathway stimulated by hypoxia. PKC has many important downstream effectors. One important target that may be crucial for proliferation is the MAPK family of enzymes. The MAPKs p44 (Erk 1) and p42 (Erk 2) are vital to proliferation in response to growth factors in a variety of cell types. The growth factors, via their cell surface receptors, initiate a series of events that culminate in the phosphorylation of inactive ras-GDP to active ras-GTP. In turn, ras activates Raf, which activates MEK (MAPKK/Erk kinase). MEK very specifically activates Erk 1 and Erk 2 by tyrosine and threonine phosphorylation. Active Erks are proline-directed, serine-threonine kinases that can phosphorylate cytoplasmic proteins and translocate to the nucleus where they activate transcription factors such as Elk-1 and genes involved in proliferation such as c-fos.

Recent work has shown that Erk-mediated signaling is important in stress-induced proliferation via H2O2 in pulmonary arterial SMC and airway SMC. This response appears to be PKC-dependent. We sought to determine whether Erk 1 and 2 mediate proliferation in pulmonary arterial adventitial fibroblasts induced by hypoxic stress. To separate the proliferative effects of hypoxia from those of growth factors and cytokines, we performed experiments in growth-arrested, serum-deprived cells. Under these conditions, hypoxia induced an increase in DNA synthesis above normoxic levels, as measured by 3H-thymidine incorporation, in pulmonary artery adventitial fibroblasts as early as 24 hours after exposure. Continued exposure to hypoxia for 3 days resulted in increased cell density compared with cultures that were maintained in normoxic atmosphere. Thus, the proliferative stimulus of hypoxia is both early and sustained. Interestingly, we found that only 25% of cultured systemic adventitial fibroblasts that were isolated from the aortas of the same animals demonstrated hypoxia-induced increases in DNA synthesis when assayed for hypoxic induction of DNA synthesis.

We also sought to determine whether the proliferative effect of hypoxia on adventitial fibroblasts was dependent on oxygen concentration by comparing levels of DNA synthesis under oxygen concentrations ranging from 1% to 20%. We found that 3% oxygen stimulated DNA synthesis maximally, and 1% oxygen did not increase DNA synthesis. Hypoxic-induced proliferation was associated with an increase in Erk 1/Erk 2 activity, as measured by the ability of immuno-complexes to incorporate 32P label onto epitHELIAL growth factor receptor peptides, a known substrate for Erk activity. Hypoxia induced a transient increase in Erk activity, peaking at 10 minutes and returning to basal levels at 30 to 45 minutes. The peak represented a 2.5-fold increase in activity that was 25% of the activity detected under maximal stimulation by serum. Transient increases in Erk activity have been reported with growth factor-induced proliferation.
of other cell types, usually peaking at 5 minutes and returning to basal at 15 minutes. Importantly, an increase in Erk activity was noted at 24 and 72 hours in cells that remained exposed to hypoxic conditions. Interruption of the Erk signaling pathway by inhibition of ras activation or MEK activation abrogated the ability of hypoxia to stimulate DNA synthesis and abolished the increase in cell density noted with sustained hypoxia under serum-deprived conditions. Thus, it appears that the ability of hypoxia to stimulate proliferation in adventitial fibroblasts under serum-deprived conditions is at least partially dependent on the Erk signaling pathway. At the moment, these findings demonstrate the role for Ca\(^{2+}\)-dependent isozymes of PKC (32 alpha and beta-II) in the augmented growth of immature fibroblasts. Therefore, we believe hypoxic-stimulated Erk activity to be at least partially dependent on PKC, a finding similar to that reported from hypoxia-induced proliferation in cardiomyocytes.

**ROLE OF APOPTOSIS IN ADVENTITIAL REMODELING**

Cell number is determined within each cell line by a balance between proliferation and death, highly regulated processes with numerous checks and balances. Apoptosis (programmed cell death) is a physiologic form of cell death. Marked increases in the rate of apoptosis have been observed in SMC in vivo during the development of intimal thickening due to balloon injury. In addition, SMC derived from human coronary atheromatous plaques exhibit a markedly elevated rate of apoptosis in vitro. Interestingly, hypoxia acts as a physiologically selective agent against apoptosis-resistant cells in tumors, thus promoting the clonal expansion of the cells that acquire mutations in their apoptotic programs during tumor development. Activation of JNK and p38 and concurrent inhibition of Erk appears to be critical for induction of apoptosis in some cells.

Preliminary studies have shown that different fibroblast subpopulations exhibit significant differences in their susceptibility to apoptosis on serum withdrawal as well as in response to hypoxia and cytokines. Susceptibility to apoptosis on serum withdrawal and in response to interferon-gamma and IL-1 alpha varies considerably among the cell subpopulations identified and in some instances appears correlated to serum-stimulated growth rate. Fast-growing subpopulations have significantly higher rates of apoptosis in response to serum withdrawal and cytokines than the slow-growing populations. It is not known whether hypoxia-sensitive subsets of fibroblasts have higher susceptibility to apoptosis or whether hypoxia can induce proliferation and apoptosis simultaneously in selective fibroblast subpopulations. The exact signaling mechanisms responsible for the induction of apoptosis in response to serum withdrawal, cytokines, and hypoxia in adventitial fibroblasts is not known.

**Clinical Correlation and Summary**

The molecular and cellular changes in the pulmonary circulation during the development of hypoxic pulmonary hypertension are complex. Pulmonary artery endothelial cells, SMC, and fibroblasts all undergo alterations of both intra- and intercellular signaling pathways, proliferation, and matrix protein synthesis in response both to hypoxia and to the changes in hemodynamic forces that global hypoxia imparts on the pulmonary arterial bed. The consequence of these complex changes is pathologic structural remodeling of the pulmonary vascular bed, which results in severe pulmonary hypertension that often is poorly or completely unresponsive to therapies and ultimately leads to cor pulmonale and death. However, as we begin to understand these complex processes better, we can develop new strategies to treat pulmonary hypertension, to avoid complications of present therapies, and ultimately to develop new therapies aimed at preventing the early cellular and molecular changes prompted by pulmonary hypertensive stimuli, such as hypoxia.

In light of our current understanding about the pulmonary hypertensive process, care must be taken to evaluate treatment strategies traditionally used to manage patients who may be exposed to hypoxia/hypoxemia in the presence of increased pulmonary blood flow. An example would be in the medical management of infants born with hypoplastic left heart syndrome. To “balance” the right ventricular output between the pulmonary and systemic circulations, many institutions now treat neonates with subatmospheric concentrations of oxygen. The resultant hypoxia increases pulmonary vascular tone, diminishes pulmonary blood flow, and allows for better perfusion of the systemic circulation. The success of this maneuver in acutely improving systemic blood pressure and perfusion and in treating acidosis is undisputed. However, over time the interaction between increased pulmonary vascular flow in the presence of increased pulmonary vascular tone due to hypoxic pulmonary vasoconstriction may result in an acceleration of pulmonary vascular structural changes. Such changes would mimic what has been described in the models of chronic hypoxic superimposed on increased pulmonary blood flow. If significant pulmonary structural changes ultimately result in pulmonary hypertension that is unresponsive to vasodilator therapies, definitive treatment of hypoplastic left heart syndrome with surgery or heart transplantation may no longer be feasible.

Unfortunately, current treatment options for severe pulmonary hypertension associated with significant vascular remodeling remains inadequate. Obviously, one problem is the frequently delayed diagnosis that leads to worsening vascular disease. Recognition of infants and children at risk of developing pulmonary hypertension is important, and evaluation of the pulmonary artery pressure is essential. Further, effective approaches to treatment must block progression of the vascular remodeling process and promote regression of established vascular changes. Circumventing the problem by the formation of new blood vessels may be another viable approach.
Fortunately, there are many potential targets for pharmacologic intervention, including extracellular matrix components, locally produced vasoregulatory and mitogenic proteins, intracellular signal transduction cascades, and cell cycle intermediates. Experimental data strongly suggest that manipulating the endogenous vascular elastases and MMP cascades initiated during the vascular remodeling process could be extremely useful at reducing and potentially even reversing the remodeling process. ET inhibitors have been particularly useful in animal models at preventing hypoxia-induced structural remodeling, but little evidence suggests that they are useful in reversing the vascular remodeling process. There also is reason to believe that ACE inhibitors or antagonists of the type I angiotensin receptor could be valuable. In addition, because entry into and progression of vascular cells through the cell cycle is considered a key event in the vascular proliferative diseases, targeting the machinery that regulates the cell cycle is one method of interrupting the “final common pathway” of many growth-promoting signals. This approach provides an attractive therapeutic tool for the prevention and perhaps even reversal of vascular proliferative diseases. These therapies have been successful at inhibiting intimal changes induced by injury in the systemic circulation, but no work has been done to date in the pulmonary circulation. It is clear that vascular remodeling must be considered when designing a therapeutic strategy for the patient who has severe pulmonary hypertension. The most promising strategies and the most favorable combinations of drugs remain to be identified.

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