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MINIREVIEW

Hypoxia-Induced Pulmonary Vascular Remodeling: Contribution of the Adventitial Fibroblasts

K. R. STENMARK[1], D. BOUCHEY[1], R. NEMENOFF[2], E. C. DEMPSEY[3], M. DAS[1]

[1]*Cardiovascular Pulmonary, Developmental Biology Research Laboratories, Denver,*
[2]*Division of Renal Medicine, University of Colorado Health Sciences Center, Denver,*
[3]*Denver Veterans Administration Medical Center, Denver, USA*

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Summary

Vascular repair in response to injury or stress (often referred to as remodeling) is a common complication of many cardiovascular abnormalities including pulmonary hypertension, systemic hypertension, atherosclerosis, vein graft remodeling and restenosis following balloon dilatation of the coronary artery. It is not surprising that repair and remodeling occurs frequently in the vasculature in that exposure of blood vessels to either excessive hemodynamic stress (e.g. hypertension), noxious blood borne agents (e.g. atherogenic lipids), locally released cytokines, or unusual environmental conditions (e.g. hypoxia), requires readily available mechanisms to counteract these adverse stimuli and to preserve structure and function of the vessel wall. The responses, which were presumably evolutionarily developed to repair an injured tissue, often escape self-limiting control and can result, in the case of blood vessels, in lumen narrowing and obstruction to blood flow. Each cell type (i. e. endothelial cells, smooth muscle cells, and fibroblasts) in the vascular wall plays a specific role in the response to injury. However, while the roles of the endothelial cells and smooth muscle cells (SMC) in vascular remodeling have been extensively studied, relatively little attention has been given to the adventitial fibroblasts. Perhaps this is because the fibroblast is a relatively ill-defined cell which, at least compared to the SMC, exhibits few specific cellular markers. Importantly though, it has been well demonstrated that fibroblasts possess the capacity to express several functions such as migration, rapid proliferation, synthesis of connective tissue components, contraction and cytokine production in response to activation or stimulation. The myriad of responses exhibited by the fibroblasts, especially in response to stimulation, suggest that these cells could play a pivotal role in the repair of injury. This fact has been well documented in the setting of wound healing where a hypoxic environment has been demonstrated to be critical in the cellular responses. As such it is not surprising that fibroblasts may play an important role in the vascular response to hypoxia and/or injury. This paper is intended to provide a brief review of the changes that occur in the adventitial fibroblasts in response to vascular stress (especially hypoxia) and the role the activated fibroblasts might play in hypoxia-mediated pulmonary vascular disease.

Key words

Vascular remodeling • Smooth muscle cells • Hypoxia • Mitogen-activated protein kinase • Protein kinase C • Extracellular matrix • Growth factors • Tyrosine kinase • Pulmonary hypertension

Fax+4202 24920590
E-mail: physres@biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

Fibroblast responses to hypoxia, vascular stress, and injury: in vivo studies

Hypoxia-induced pulmonary hypertension complicates the clinical course of many important pulmonary and cardiac diseases in children and adults (Jones and Reid 1995, Stenmark *et al.* 1995, Stenmark and Mecham 1997). The pulmonary hypertension and accompanying structural remodeling appear particularly severe in infants and it is in this stage of life where adventitial changes often predominate. For instance, even the earliest pathologic descriptions of persistent pulmonary hypertension of the newborn (PPHN) pointed out significant adventitial thickening (Murphy *et al.* 1981). Dramatic adventitial changes have also been observed in the lung vessels of young infants dying of high-altitude-induced pulmonary hypertension (Siu *et al.* 1988). Similar, though less dramatic, changes are seen in the adventitial compartment of infants with cyanotic forms of congenital heart disease which are complicated by pulmonary hypertension.

In animal models, the earliest and most dramatic structural changes following hypoxic exposure are found in the adventitial compartment of the vessel wall (Jones and Reid 1995, Stenmark *et al.* 1995, Stenmark and Mecham 1997). Resident adventitial fibroblasts have been shown to exhibit early and sustained increases in proliferation that exceed those observed in endothelial or smooth muscle cells (Belknap *et al.* 1997, Meyrick and Reid 1979). In addition, early and dramatic changes in extracellular matrix protein synthesis occur (Durmowicz *et al.* 1994). These include early upregulation of collagen, fibronectin, and tropoelastin mRNAs followed by the subsequent deposition of each of these proteins. These changes in the proliferative and matrix producing phenotype of the fibroblast are accompanied by appearance of α-SMC-actin, in at least some of the cells in the adventitial compartment, indicating a phenotypic modulation of some fibroblasts to myofibroblasts (Stenmark *et al.* 1995). The fibroproliferative changes in the adventitia are ultimately associated with luminal narrowing and progressive decrease in the ability of the vessel wall to respond to vasodilating stimuli (Durmowicz *et al.* 1993).

The fibroproliferative changes described in the adventitial compartment are observed at all levels along the longitudinal axis of the pulmonary artery. However, it is in small muscular pulmonary arteries where the magnitude of change is greatest. We have also documented that rapid medial thickening also occurs in these vessels. Since the media of small muscular pulmonary vessels is composed of an apparently homogeneous population of highly differentiated SMC with low growth potential (Gnanaskharan *et al.* 2000) (compared to the heterogeneous composition of elastic arteries), the possibility that fibroblast-like cells were also recruited into the media was investigated. Using explant techniques, we found that only populations of highly differentiated, minimally growth responsive SMC could be isolated confirming previous observations of SMC homogeneity in small muscular arteries. However, two populations of cells were consistently isolated from the media of small pulmonary arteries of chronically hypoxic animals. One population was similar to the SMC cultured from control vessels, the other was a small stellate-like cell that expressed α-SMC-actin but not myosin. This cell was extremely growth responsive and in fact proliferated under hypoxic conditions in the absence of exogenous growth factors. This response has only been described in adventitial fibroblasts or in the non-muscle epitheloid-like cells previously described in the large vessels (Das *et al.* 2000, Frid *et al.* 1997). This cell also exhibited enhanced migratory properties compared to control SMC or even fibroblasts from the adventitia. Migration was enhanced under hypoxic conditions and found to be in part due to upregulation of matrix metalloproteinase (MMP)-2 under normoxic and hypoxic conditions. Thus observations in several systems are consistent with the hypothesis that activated fibroblasts migrate into the media and perhaps intima in response to certain injurious stimuli and suggest a role for fibroblasts in the medial as well as adventitial changes observed in small vessels in numerous forms of pulmonary hypertension.

The idea raised by these observations, that hypoxia acts directly or in unique ways on the adventitial fibroblast (compared to SMC or endothelial cells) is raised by previously well-documented observations that reduced oxygen tension (anoxia/hypoxia) results in profound changes in fibroblast physiology and metabolism. Cellular anoxia represents a biochemical state distinct from hypoxia yet still represents a normal physiological condition present during wound healing and perhaps vascular disease (Anderson *et al.* 1995). Responses that differ from those seen with hypoxia are observed in anoxic fibroblasts suggesting that these cells must possess the unique ability to activate different,

possibly overlapping sets of genes to cope with these unique environmental conditions (Anderson *et al.* 1989). The activity of several different transcription factors is known to be influenced by low oxygen tension. In cells which are stressed by oxygen deprivation, NF-κ B activity increases as a result of phosphorylation and subsequent degradation of IκBα (Koong *et al.* 1994). In other cells, low oxygen tensions induce the transcription of multiple members of bZIP (basic/leucine zipper domain) superfamily (Ausserer *et al.* 1994, Estes *et al.* 1995b, Yao *et al.* 1994), result in nuclear accumulation of p53 (Graeber *et al.* 1994), or induce the activity of hypoxia-inducible factor 1 (HIF-1) (Semenza and Wang 1992). Estes *et al.* (1995a) demonstrated that one DNA binding activity induced in hypoxic, anoxic, and cobalt-treated fibroblasts recognizes secondary anoxia-responsive element (SARE) and has electrophoretic mobility similar to that of HIF-1. It has also been demonstrated that a second, more prominent SARE binding activity, termed the anoxia-inducible factor (AIF), is also induced in anoxic fibroblasts. Two-dimensional gel analysis indicated that AIF is a heterodimer composed of 61- and 52-kDa subunits and is likely to arise from post-translational modification of a heterodimeric SARE binding complex present in aerobic cells. Identification of a mammalian anoxic response element has interesting implications. This element may prove to be useful in gene therapy regimens for targeting expression to physiological situations in which functional anaerobiosis exists, such as during wound healing. Interestingly, deregulation of genes normally expressed during anoxia is often also seen in cancer cells regardless of their state of oxygenation (Anderson *et al.* 1989). Thus, understanding the molecular basis of the mammalian hypoxic and anoxic regulatory pathways in fibroblasts may lead to unique approaches to therapeutic intervention in settings of vascular disease complicated by hypoxia.

Based on the above, one might suspect that fibroblast changes in vascular disease are unique to conditions or models involving hypoxia. However, 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 has been demonstrated to occur in adventitial fibroblasts of the coronary vasculature following balloon catheter-induced injury that is similar to that seen in the skin wound healing process (Scott *et al.* 1996, Serini and Gabbiani 1999, Shi *et al.* 1996a,b,c, 1997a,b). Cell proliferation is an early phenomenon and may involve the entire adventitia of the blood vessel (Shi *et al.* 1996c). 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 have been shown to differentiate into myofibroblast-like cells with the appearance of α-SMC-actin in adventitial cells beginning as early as 3 days and reaching a maximum at 14 days (Shi *et al.* 1996b). These changes in proliferation and contractile protein expression in adventitial cells are accompanied by the induction of procollagen α-1 mRNA and subsequent protein accumulation in the adventitial compartment (Shi *et al.* 1997a). Additionally, recent studies also suggest that these activated fibroblasts (? myofibroblasts) may migrate through the vessel wall and be at least partially responsible for the intimal thickening which ultimately characterizes the coronary vasculature following balloon injury (Serini and Gabbiani 1999, Shi *et al.* 1996a). Fibroblast recruitment into the media of the vessel wall is also observed in the setting of hyperoxic-induced pulmonary hypertension where fibroblasts are speculated to be the "source" of SMC in newly muscularized vessels (Jones and Reid 1995). Similar properties have been attributed to the fibroblasts in the vascular remodeling observed in venous grafts following placement in the arterial system (Shi *et al.* 1997b).

Of relevance to this discussion is the suggestion that one mechanism involved in the adventitial induction of neointimal proliferation involves obstruction of the vasa vasorum with subsequent vascular wall hypoxia (Gutterman 1999). For example, external stenting of vein grafts is associated with interruption of adventitial perfusion and resultant neointimal formation. This is prevented by the use of porous external stents, which allow microangiogenesis, thereby minimizing graft hypoxia (Jeremy *et al.* 1998). Other mechanisms, particularly those related to hemodynamics, may also contribute to adventitial cell activation and proliferation. It has been shown that chronic systemic dilator treatment with prazosin leads to an eightfold increase in arteriolar adventitial fibroblast proliferation (Price and Skalak 1998). DNA synthesis is increased 600 % in adventitial fibroblasts proximal to an aortic obstruction in a rat model of hypertension (Chatelain and Dardik 1988) suggesting that fibroblasts may also be activated by increases in arterial pressure. Thus, observations in both the pulmonary and systemic circulation suggest that the adventitial fibroblast can respond vigorously and quickly

to a variety of pathophysiologic stimuli and ultimately plays a major role in the vascular repair process.

Fibroblast interactions with other cell types: role in the hypertensive process

A large body of experimental evidence demonstrates that fibroblasts exert significant paracrine effects on other cell types raising the possibility that they contribute to the vascular remodeling process in dynamic ways which are additive to direct changes in their phenotype. In fact, the idea that dynamic and reciprocal relationships exist between fibroblasts and other cell types is well documented, especially in the developmental biology (Wessells 1997). Interactions between epithelium and mesenchyme have been shown to be critical for the morphogenesis of many different organs including the lung (Shannon and Deterding 1997). Studies in both avian and mammalian species have demonstrated that an interaction of the presumptive lung epithelium with lung mesenchyme is absolutely required for normal branching morphogenesis to proceed. Heterotypic recombination experiments have indicated that the morphogenesis and cytodifferentiation of the epithelial component may be determined by the site of origin of the mesenchyme with which it is recombined. Conversely, epithelial cells may influence various important aspects of fibroblast function such as matrix deposition and secretion of matrix degrading enzymes.

The biochemical identity of the signal molecules mediating mesenchymal-epithelial interactions, has been intensively investigated (Minoo and King 1994, Shannon and Deterding 1997). It has been established that matrix proteins such as collagen, fibronectin and proteoglycans play a prominent role. As suggested by many others, there is continuous feedback of information between cell and matrix (see Fig. 1). Interaction of specific matrix molecules with their receptors at the cell surface is involved in a diverse array of cell behaviors including migration, proliferation and differentiation. With regard to the role of fibroblasts in pulmonary vascular disease, it is important to note that it has recently been determined that activation of the myofibroblasts requires the presence of the ED-A domain of fibronectin. It has been shown this fibronectin ED-A domain is necessary for transforming growth factor (TGF)-β to trigger α-SMC-actin expression and collagen secretion by myofibroblasts (Serini and Gabbiani 1999). Of note is the fact that fibronectin mRNA is highly expressed by adventitial fibroblasts *in vivo* within the first 24 h of exposure to hypoxia thus potentially setting the stage for fibroblast activation and subsequent interaction with neighboring SMC or endothelial cells.

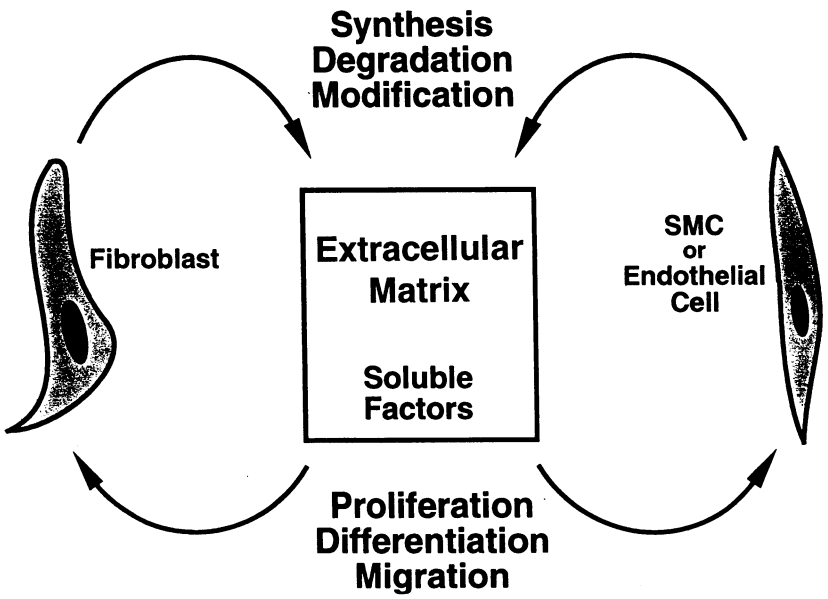

Fig. 1. *Dynamic reciprocity in vascular wall cell interactions. Changes in the production of extracellular matrix or soluble factors by one cell type may have profound effects on the phenotype of other cells in the vascular wall.*

Fibroblasts are known to produce a wide array of cytokines, growth factors and inflammatory mediators which function as autocrine regulators of fibroblast function and paracrine regulators of neighboring cell (endothelial, SMC, epithelial) proliferation, migration, and biosynthetic activity. The biological activity of the

soluble factors and the nature of the extracellular matrix (ECM) in contact with the cells are mutually interdependent in that soluble factors (e.g. TGF-β, epithelial growth factor, insulin-like growth factor) exert effects on matrix biosynthesis and the response of cells to these factors is modulated by the nature of the matrix (Cardoso 1995, Minoo and King 1994) (Fig. 1). In addition to the release of traditional growth factors, recent observations suggest that the adventitia may be a particularly rich source of reactive oxygen species. Superoxide anion production occurs endogenously in aortic tissue and is derived primarily from NADPH oxidase in the adventitia (Pagano *et al.* 1997, Wang *et al.* 1998). In fact, Wang *et al.* (1998) demonstrated that superoxide anion from the rat thoracic aorta inactivates nitric oxide. In an animal model of systemic hypertension superoxide anion production is elevated 15-fold, with the majority arising from adventitial fibroblasts (Di Wang *et al.* 1999). The excess of superoxide anions was causally related to spontaneous oscillations in vessel tone and possibly contributed to the hypertension. In addition, interaction of NO and superoxide anion results in the production of peroxynitrite, which may itself cause endothelial dysfunction and increase vascular tone. Thus, it seems possible that chronic hypoxia could be associated with increased radical production in the adventitia, leading to inactivation of NO and alterations in pulmonary vascular tone.

Experiments in other vascular injury models also demonstrated the importance of fibroblast communication with other vascular wall cells. For instance, application of the inflammatory cytokine interleukin-1 β to the adventitia induces coronary vasospasm and neointimal formation even without endoluminal manipulations (Shimokawa *et al.* 1996). These findings bear relevance to clinical settings since the accumulation of mast cells and an inflammatory reaction are notable in patients with coronary vasospasm and fatal unstable coronary syndromes, respectively (Kohchi *et al.* 1985, Kolodgie *et al.* 1991). Similarly, molecules which can inhibit cellular proliferation applied to the adventitia have been shown to decrease the development of intimal thickening in response to luminal injury (Andersen *et al.* 1996, Booth *et al.* 1989, Rogers *et al.* 1993). Thus, a substantial body of *in vivo* and *in vitro* evidence suggests a dynamic reciprocity in the interaction between adventitial fibroblasts and other vascular wall cells similar to the dynamic interactions well documented between mesenchymal cells and epithelium during development.

Developmentally regulated changes in fibroblast growth potential: increased sensitivity to hypoxia and injury in immature fibroblasts

Pathologic studies suggest the pulmonary artery adventitial response to chronic hypoxia is greater in infants and children than in adults. Observations suggesting that developing blood vessels often exhibit heightened or unique responses to injurious stimuli compared to mature vessels is supported by *in vitro* studies demonstrating that SMC and fibroblasts isolated at different developmental stages exhibit unique biochemical and functional characteristics. Several studies have demonstrated that SMC derived from embryonic, neonatal and adult animals exhibit remarkably different growth potential (Dempsey *et al.* 1994, Stenmark *et al.* 1995, Stenmark and Mecham 1997, Xu *et al.* 1997). In general, a high growth potential has been associated with SMC derived from the developing vasculature. Similarly, fetal fibroblasts have been shown to display a number of behavioral and biochemical characteristics not normally expressed by their adult counterparts. These include elevated production of unique soluble growth and transforming peptide factors, the synthesis of particular species and/or isoforms of matrix macromolecules, the presence of fetal-specific antigenic determinants, and production of several migration stimulating factors (Schor and Schor 1987). Programmed fetal to adult transitions in the above characteristics occur at various times during development, including the neonatal period, and appear to play an important role in the control of both the normal developmental process and the response to injury. Importantly, re-expression of fetal or neonatal-like characteristics and genes have been observed in SMC and fibroblasts following vascular injury in adult animals (Stenmark and Mecham 1997).

Within the pulmonary circulation we wanted to determine whether there were differences in the growth capacity of fibroblasts isolated from the pulmonary artery at different stages of development. We therefore measured the rate of fetal, neonatal and adult fibroblast proliferation in 10 % serum containing media. We found that fetal and neonatal fibroblasts had increased DNA synthesis, grew faster, and reached higher plateau densities than adult cells (Das *et al.* 1995). The earlier during gestation the fetal fibroblasts were harvested, the more rapid was the growth observed. Serum deprived fetal fibroblasts had increased DNA synthesis in response to a panel of potentially relevant mitogens, including

phorbol myristate acetate (PMA), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor, and the combination of PMA + IGF-1 compared with adult cells.

Little is known regarding the cellular mechanisms that might confer unique growth and differentiation properties to fibroblasts at various developmental stages. Protein kinase C (PKC) is a signaling pathway which has been shown to be important for both pulmonary and non-pulmonary vascular cell growth (Johannes *et al.* 1995, Nishizuka 1992). This pathway is known to be developmentally regulated and has been shown to contribute specifically to the enhanced growth properties previously described for fetal and neonatal pulmonary artery SMC (Xu *et al.* 1997). Increased expression of PKC has also been associated with enhanced growth capabilities of nonvascular fibroblasts. We therefore investigated the possibility that the augmented growth response to serum and peptide mitogens of fetal and neonatal fibroblasts compared to adults would be in some way related to changes in PKC signaling. It was established first that rapidly growing fetal fibroblasts had increased whole cellular PKC catalytic activity compared to adult cells (Das *et al.* 1995). Then, using three different PKC inhibitor strategies, it was also documented that high rates of growth in fetal cells were due in part to high levels of PKC pathway activity since growth of fetal and neonatal fibroblasts was decreased by the inhibitors to a far greater extent than that of adult cells (Das *et al.* 1995).

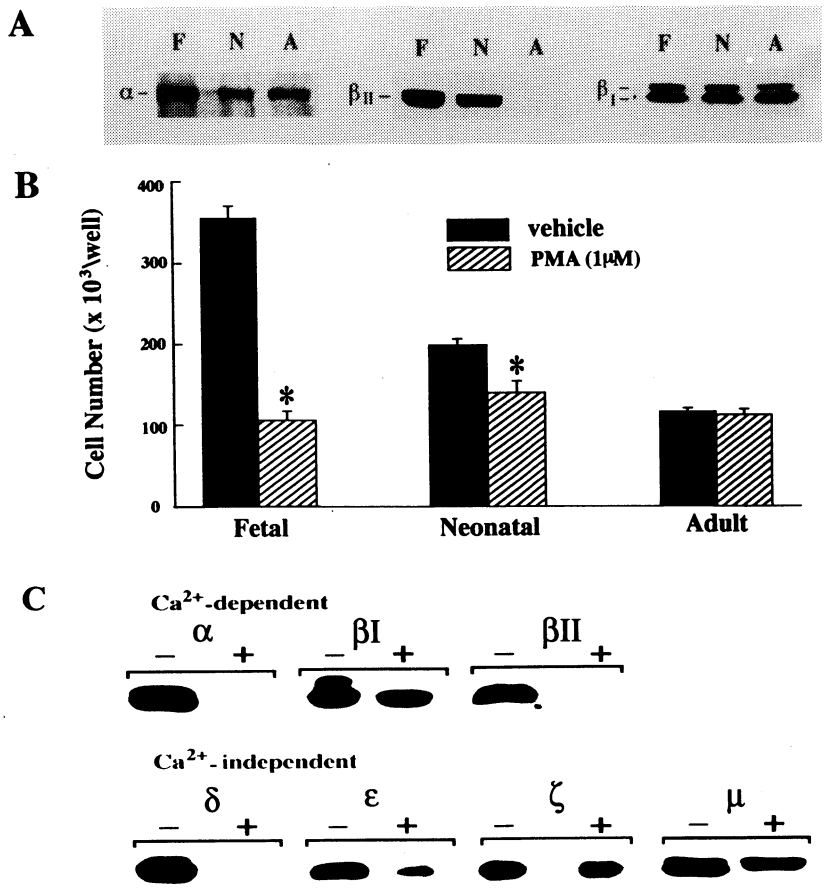

Fig. 2. *Expression of the Ca^{2+}-dependent α and βII isozymes of PKC is higher in immature pulmonary artery adventitial fibroblasts than in adult cells and contribute to their augmented growth properties. A) Representative immunoblots for each Ca^{2+}-dependent isozyme. Whole cell lysates of fetal (F), neonatal (N), and adult (A) pulmonary artery adventitial fibroblasts were resolved by SDS-PAGE, transferred to nitrocellulose, probed with anti PKC-α, βI, and βII antibodies. B) Pretreatment with 1 μM phorbol 12-myristate 13-acetate (PMA) for 24 h inhibits growth of immature pulmonary artery adventitial fibroblasts; C) Treatment of cells for 24 h with PMA downregulates PKC-α and PKC-βII but not PKC-βI; n = 4 replicate wells. *p<0. 05 compared with control cells. Reproduced from Das et al. (1997b).*

The PKC signaling pathway, however, is a complex one, with eleven isozymes of PKC having been identified and divided into three distinct categories: 1) conventional (α, βI, βII, γ); 2) novel (δ, ε, η, θ); 3) atypical (ζ, ι, μ) (Johannes *et al.* 1995, Nishizuka 1992). Conventional isozymes are considered to be calcium-dependent, whereas novel and atypical isozymes are calcium-independent. Developmental changes in the expression of individual isozymes have been observed and increased expression of selected PKC isozymes has been linked to augmented growth capacity. We therefore sought to determine specifically the isozymes of PKC that might be contributing to the developmental differences in growth exhibited by pulmonary artery

adventitial fibroblasts. We detected three calcium-dependent (α, βI, βII) and four calcium-independent (δ, ε, ζ, μ) PKC isozymes in fibroblasts derived from the neonatal pulmonary artery (Das *et al.* 1997b). We then demonstrated a selective increase in the expression of two calcium-dependent isozymes of PKC: α, and βII, but not βI, in immature fibroblasts which paralleled the developmental differences in growth and PKC catalytic activity of immature pulmonary artery fibroblasts (Fig. 2A). Then, again using pharmacologic antagonist strategies, we demonstrated that these same two isozymes, PKCα and βII were involved in the enhanced growth of fetal and neonatal fibroblasts (Figs 2B and 2C).

Hypoxia exerts bifunctional effects on adventitial fibroblast growth and death

Vascular structure is determined in part by a coordinate regulation of cell growth and death. As discussed above dramatic fibroproliferative changes in the adventitia of pulmonary arteries have been observed in chronic hypoxic pulmonary hypertension. In addition to the marked increases in proliferation that have been documented using bromodeoxyuridine labeling, we have recently demonstrated that a significant increase in the number of apoptotic cells is also observed in the adventitia of chronically hypoxic animals. This balance between proliferation and apoptosis is similar to that observed during the development of the neointima following balloon-catheter induced arterial injury (Han *et al.* 1995). Though many factors may be operational in contributing to adventitial fibroblast growth and death in the setting of chronic hypoxic pulmonary hypertension (e.g. hemodynamic forces, leak of plasma proteins, upregulation of cytokines and growth factors etc.), we were particularly interested in the effects of hypoxia alone on fibroblast proliferation and apoptosis because of its previously described effects on gene expression and its ability to induce growth in pulmonary and non-pulmonary fibroblasts, an effect not observed in most traditional SMC lines. We therefore sought to determine whether hypoxia alone is capable of stimulating proliferation in pulmonary arterial adventitial fibroblasts and to determine the role of mitogen-activated protein (MAP) kinases in these responses because of their well-known effects on proliferation and apoptosis.

In order 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 ^{3}H-thymidine incorporation, in pulmonary artery adventitial fibroblasts as early as 24 h after exposure. Continued exposure to hypoxia for three days resulted in increased cell density compared to cultures maintained in normoxic atmosphere (Bouchey *et al.* 1998). This hypoxia-induced proliferative response of bovine pulmonary artery adventitial fibroblasts is also supported by the observation of Welsh *et al.* (1998). This stimulation of growth was not observed in traditional SMC under the same conditions. Thus, the proliferative stimulus of hypoxia is both early and sustained. Interestingly, we found that when systemic adventitial fibroblasts, isolated from the aortas of the same animals, were assayed for hypoxic induction of DNA synthesis, only 25 % of the cultures demonstrated hypoxia-induced increases in DNA synthesis. In pulmonary artery fibroblasts, exposure to 3 % hypoxic conditions was also associated with a 2-fold increase in apoptotic rate. When cells were exposed to more severe hypoxic conditions (0 %), proliferation was decreased and apoptosis increased.

Since MAP kinases Erk 1/Erk 2 have been shown to play a critical role in cell proliferation, we began by assessing the effects of hypoxia on Erk 1/Erk 2. Hypoxia-induced proliferation was associated with an increase in Erk 1/Erk 2 activity, as measured by ability of immuno-complexes to incorporate ^{32}P label into specific Erk substrate. Hypoxia induced a transient increase in Erk activity, peaking at 10 min and returning to basal levels by 60 min. The peak represented a 2.5-fold increase in activity, which was 25 % of the activity detected under maximal stimulation by serum. When assessed by antibodies directed against phosphorylated and activated Erks, a similar time course of activation was observed, although differences in phosphorylation of Erk 1 and Erk 2 were noted. We demonstrated that interruption of the Erk signaling pathway, both by inhibition of *ras* activation or inhibition of MEK activation, abrogated the ability of hypoxia to stimulate DNA synthesis and also abolished the increase in cell density noted with sustained hypoxia under serum-deprived conditions. Transient increases in Erk activity have been reported with growth factor induced proliferation of other cell types, usually peaking at 5 min and returning to basal at 15 min. Importantly, a rise in Erk activity was again noted at 24 h in cells that remained exposed to hypoxic conditions. Interestingly, inhibition of Erk had no effect on apoptotic activity in response to 3 % O_2. 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.

Other members of the MAP kinase family, including JNKs and p38, may mediate proliferative as well as cell death responses and therefore their responses and role in proliferation and apoptosis in response to hypoxia were evaluated. We found that hypoxia stimulated a transient increase in JNK activity as measured by phospho-specific antibodies which peaked at 10 min and disappeared by 30 min. As opposed to Erk 1/Erk 2, no secondary increase in JNK was noted at 24 h. We then used antisense oligonucleotides to JNK 1 and 2 to evaluate the role of JNK activation in hypoxia-mediated proliferation and apoptosis. JNK-1 antisense oligonucleotides attenuated proliferation while JNK-2 antisense oligos had no effect. On the other hand, JNK-2 antisense oligos inhibited hypoxia-induced apoptosis while JNK-1 oligos had no effect. Lastly, we found that hypoxia (3 % O_2) also activated p38. Peak phosphorylation was observed at 10 min in a time course similar to that observed for JNK. Hypoxia-induced activation of p38 MAP kinase in adventitial fibroblasts is consistent with the observation of Scott *et al.* (1998). When p38 was inhibited with SB 202190, an attenuation of hypoxia-induced proliferation was observed. We therefore conclude that hypoxia-induced adventitial remodeling is a net result of both proliferative and apoptotic responses mediated in part by a highly complex network of MAP kinases (see Fig. 3).

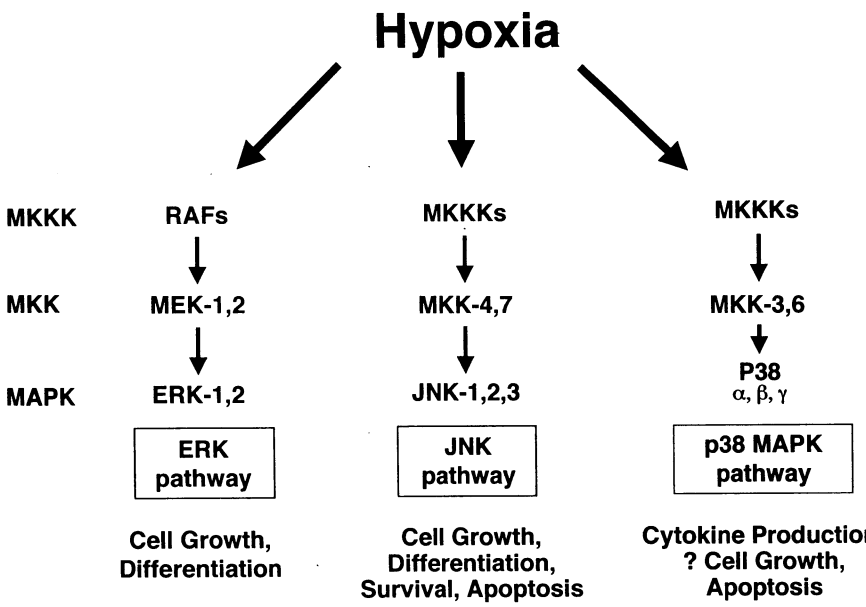

Fig. 3. *Mitogen-activated protein kinase pathways is activated by hypoxia. It is hypothesized that the final cellular response is dependent on the severity and length of hypoxic exposure and the downstream effectors of MAP kinase available in specific cell subpopulations.*

Chronic hypoxic exposure induces changes in fibroblast phenotype

In addition to the developmentally regulated changes in the proliferative and matrix producing potential of fibroblasts, an expanding body of experimental observations also supports the concept that significant changes in the phenotypic properties of resident fibroblasts can occur in the setting of fibroproliferative disease (Chen *et al.* 1992, Jordana *et al.* 1988, 1992, Krieg and Meurer 1988, Lukacs *et al.* 1994, Raghu *et al.* 1988, Rodemann and Muller 1990, Torry *et al.* 1994). Phenotypically altered fibroblasts might contribute significantly to the generation and maintenance of the fibroproliferative response observed in chronic vascular remodeling. For example, stable increases in the proliferative capacity of mesenchymal cells have been observed in the affected organs of patients with atherosclerosis, progressive systemic sclerosis, idiopathic pulmonary fibrosis, interstitial renal fibrosis and hepatic fibrosis (Chen *et al.* 1992, Jordana *et al.* 1988, 1992, Krieg and Meurer 1988, Lukacs *et al.* 1994, Raghu *et al.* 1988, Rodemann and Muller 1990, Torry *et al.* 1994). In animal models of lung and renal fibrosis, increased proliferative capacity of fibroblasts isolated from fibrotic tissue has also been demonstrated. The phenotype has been observed to be stable for many population doublings suggesting significant and persistent changes that become intrinsic to the cell itself (i.e. not dependent on changes in the local environment from which the cell was isolated). Little is known, however, of the stimuli responsible for inducing changes

in fibroblast phenotype or the changes in intracellular signaling that might occur during this process.

We therefore performed studies to address the hypothesis that exposure to chronic hypoxia would induce specific alterations in resident fibroblasts rendering them more sensitive to growth promoting mitogens as well as to hypoxia. To address the question, adventitial fibroblasts from age-matched control and chronically hypoxic calves were isolated simultaneously and subsequent comparative experiments were performed in cells from the different animals under identical conditions. We found, on a very consistent basis, that fibroblasts isolated from hypoxic hypertensive calves exhibited augmented growth responses to serum, hypoxia, and purified peptide mitogens compared to those obtained from age-matched control animals (Das *et al.* 2000). Importantly, a synergistic interaction between hypoxia and purified peptide mitogens was observed in fibroblasts, isolated from the hypertensive animals, that was not observed in fibroblasts from the control animals (Fig. 4). These proliferative attributes persisted through numerous cell passages in culture suggesting acquired differences in fibroblast populations, which were not simply due to changes in the *in vivo* cellular milieu i.e. growth factor, cytokines and matrix components (Das *et al.* 2000).

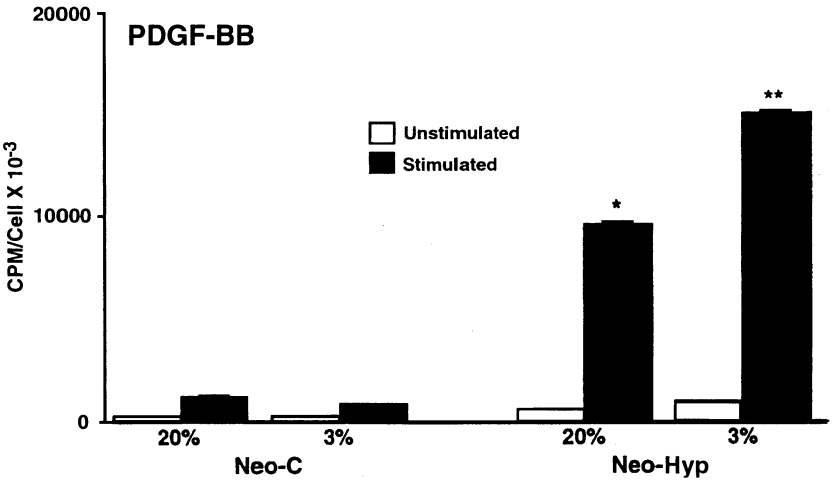

Fig. 4. *Pulmonary artery adventitial fibroblasts isolated from animals with hypoxic pulmonary hypertension (Neo-Hyp) demonstrate higher [^{3}H] thymidine incorporation following stimulation with PDGF under both normoxic and hypoxic conditions compared to the cells isolated from control animals (Neo-C). Open bars, unstimulated and solid bars, stimulated values. n = replicate wells. *p<0. 05 compared with results from control cells. Reproduced from Das et al. (2000).*

Since changes in PKC isozyme expression occur during development and appear to participate in conferring selective growth differences to different cell populations, studies were performed to address the hypothesis that fibroblasts from chronically hypoxic animals would demonstrate a change in PKC isozyme expression and utilization that would be associated with their enhanced growth properties. The differences in the utilization of specific PKC isozymes between fibroblasts from chronically hypoxic and control calves were found using PKC isozyme nonselective and selective inhibitor strategies and immunoblot analysis. PKC-βI and PKC-ζ isozymes were identified as key contributors to the enhanced growth of fibroblasts from the chronically hypoxic animals (Fig. 5). The PKC-βI isozyme appeared particularly important for the augmented growth capacity observed in fibroblasts from the hypertensive vessel wall under hypoxic conditions. This is particularly interesting because PKC-βI was not implicated in the enhanced growth capabilities of fetal compared to adult cells. These findings are compatible with those in non-vascular fibroblasts also demonstrating that PKC-βI and PKC-ζ are important in proliferative responses. Interestingly, among the PKC genes, the PKC-β gene and its gene products PKC- βI and βII are subject to the most extensive regulation, with both tissue-specific and developmental regulation being documented. Overexpression of PKC- βI in vascular SMC has been shown to increase the rate of cell proliferation and to accelerate the entry of the cells into S phase (Yamamoto *et al.* 1998). PKC-ζ has also been implicated in the mitogenic response of non-vascular and vascular cells (Zhou *et al.* 1994). PKC-ζ is particularly abundant in fetal tissues consistent with the notion that PKC-ζ plays a key role in highly proliferative cells. Thus, our observations are consistent with the hypothesis that chronic hypoxia can induce stable increases in at least the proliferative capacity of fibroblasts and that these changes result partially from changes in specific PKC isozyme and activation patterns.

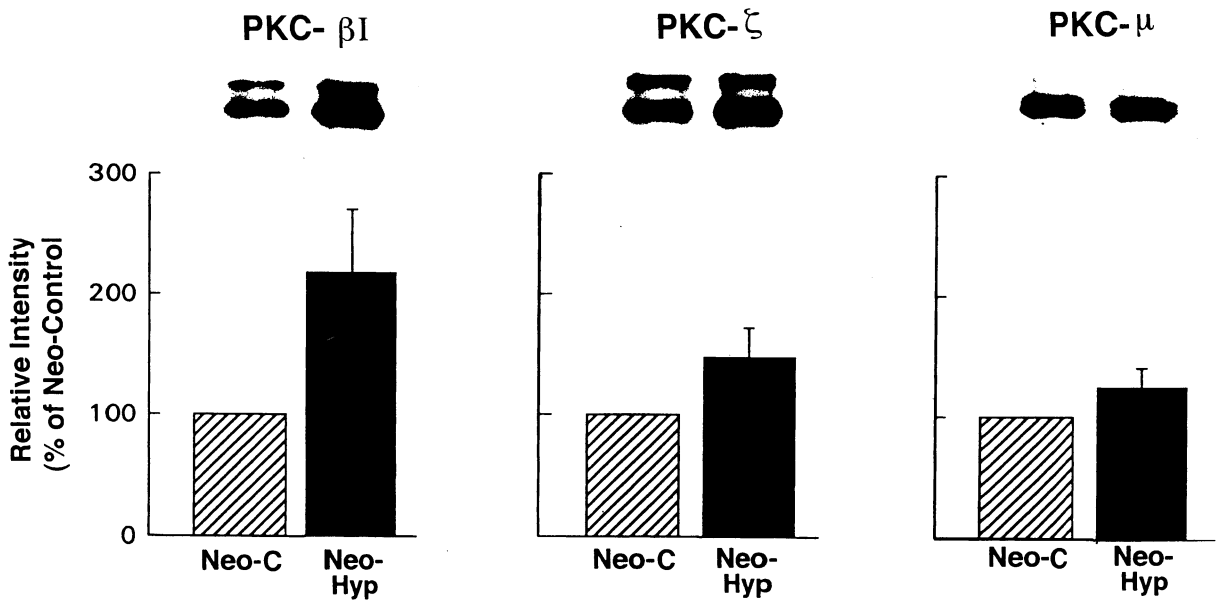

Fig. 5. *Expression of PKC-β1 and PKC–ζ are higher in fibroblasts isolated from neonatal hypoxic hypertensive calves (Neo-Hyp) than in cells of neonatal control calves (Neo-C). A: Representative immunoblots for PKC-βI, ζ and μ isozymes. B: Quantitative analysis of expression pattern for each isozyme. Reproduced from Das et al. (2000).*

Contribution of fibroblast heterogeneity to vascular remodeling

Several possibilities must be considered in explaining how a stable phenotypic alteration of the adventitial fibroblast might come about. The first is that a large portion of resident fibroblasts are altered by changes which occur locally in the chronically hypoxic vascular wall. Hypoxia itself, a combination of hypoxia with subsequent hemodynamic changes and ultimately the combination of hypoxia, altered hemodynamics and changes in the local concentrations of cytokines, growth factors and matrix proteins must be considered. Resident fibroblasts could be altered by these signals, conferring upon them a new stable phenotype, which is manifested by intrinsically enhanced proliferative and matrix producing capabilities. Another possibility is that selective expansion of a normally resident fibroblast population, with unusual or augmented growth properties, has occurred *in vivo*. This population could expand to the point where it is numerically the most important constituent of the activated or injured vessel wall. This population might then demonstrate selective advantage when cell cultures are done and thus account for the apparent phenotypic change observed in fibroblast populations from the injured organ or vessel (Fig. 6).

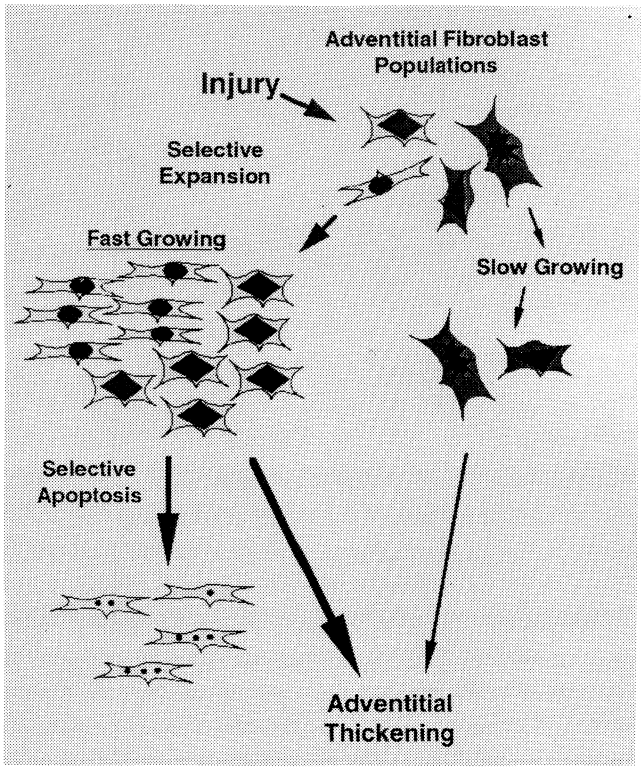

Fig. 6. *Hypothesis: Numerous heterogeneous adventitial fibroblast subpopulations exist. These cells have different intrinsic growth and matrix producing capabilities. Fibroblast subpopulations with high growth potential may contribute selectively to adventitial thickening in response to injury. However, excessive proliferation is balanced by an increased rate of apoptosis in select fibroblast subpopulations.*

In addition to the developmentally regulated and site-specific differences in fibroblast phenotype that have been well documented, there is a growing body of literature documenting the existence of phenotypic heterogeneity within the resident fibroblast population of a given tissue at a specified developmental stage. The extensive diversity of this intra-site heterogeneity includes differences in proliferative potential, synthesis of matrix molecules, production and response to growth factors, synthesis of matrix degrading enzymes, phagocytic activity and the presence of specific receptors and/or antigenic determinants at the cell surface (Fries *et al.* 1994, Phipps 1992).

To examine the possibility that several unique pulmonary artery adventitial fibroblast populations exist and thus contribute in unique ways to the vascular remodeling process, a limited dilution and clonal expansion strategy was used to try and establish pure stable subpopulations of adventitial fibroblasts. We found that subpopulations of fibroblasts could be distinguished not only on the basis of their light microscopic appearance (i. e. rounded or epitheloid vs. traditional elongated spindle shape), but also on the basis of their cytoskeletal protein expression. Subpopulations with a rounded appearance did not express α-SMC-actin in culture (Das *et al.* 1998). In contrast, there was significantly brighter immunostaining with SMC specific α-actin antibody in fibroblast subpopulations demonstrating a spindle-like appearance. Marked differences in proliferative potential of different fibroblast clones were also documented. In general, cells exhibiting a spindle-like appearance tended to demonstrate a far greater growth potential to serum or peptide mitogens than did the rounded cells. Certain clones of fibroblasts also expressed extremely high levels of type I and III collagen mRNA compared to other clones. In most, but not all instances, cells that exhibited a high growth potential also exhibited a high potential for collagen synthesis. On the other hand, elastin mRNA levels were significantly elevated in only one relatively slow growing clone of adventitial fibroblasts.

We also sought to determine if differences in the proliferative and apoptotic responses to hypoxia existed between fibroblast clones. We found that there are subsets of fibroblasts, which are highly sensitive to growth promoting effects of hypoxia, and subsets, which are resistant (Das *et al.* 1997a). Likewise, significant differences in hypoxia-initiated apoptosis were also observed between various fibroblast clones. Susceptibility to the growth or apoptotic effects of hypoxia was not predictable on the basis of morphology or growth potential in serum. In this light and perhaps relevant to the observations in fibroblasts from chronically hypoxic animals, are observations that hypoxia acts as a physiologically selective agent against apoptosis-competent cells thus promoting clonal expansion of cells resistant to hypoxia-induced apoptosis (Graeber *et al.* 1996). Thus, *in vitro* findings strongly support the idea that distinct subpopulations of fibroblasts exist within the pulmonary artery adventitia and may contribute selectively to the adventitial thickening observed in response to chronic hypoxia.

Changes in the relative proportion of different fibroblast subsets may have profound effects not only on the adventitial remodeling but on the whole vessel wall. It is possible that alteration in the relative proportion of different fibroblast subsets with different capabilities for matrix protein and soluble growth factor production, might be expected to influence the behavior of adjacent SMC or endothelial cells (dynamic reciprocity, see above). It is clear that there is continuous feedback of information between cell and matrix such that the extracellular matrix in contact with the cells is itself a product of cellular activity (by virtue of biosynthesis, degradation, and modification of matrix macromolecules), and that the extracellular matrix influences various fundamental aspects of cell behavior such as the deposition of matrix itself, cell proliferation and the pattern of gene expression and cell migration. Activation of a fibroblast subset secreting unique matrix molecules and soluble factors may thus have an influence on neighboring SMC or epithelial cells that is not exhibited under normal conditions.

Conclusions

Substantial experimental evidence supports the idea that the fibroblast may play a significant role in the vascular response to injury especially under hypoxic conditions. The cells posses the capability to rapidly respond to stress and to modulate its function in order to adapt rapidly to local vascular needs. It appears uniquely equipped to proliferate and migrate under hypoxic conditions with the proliferative responses to hypoxia apparently due to interactions between PKC and MAP kinase family members that do not occur in the vast majority of vascular SMC. The apoptotic pathways are also initiated under hypoxic conditions that appear to limit or control to some degree the fibroproliferative responses initiated by hypoxia. It is also becoming clear

that, as with SMC, numerous fibroblast subtypes exist in the vessel wall and each may serve special functions in response to injury. Future work is needed to determine more precisely the role of the fibroblasts in the wide variety of vascular complications observed in many human diseases as well as the genes and gene products that confer unique properties to this important vascular cell.

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Reprint requests
Kurt R. Stenmark, MD, Professor of Pediatrics, University of Colorado Health Sciences Center, 4200 E. Ninth Avenue, Box B131, Denver, CO 80262, USA, Fax (303) 315-8353, E-mail: Kurt.Stenmark@UCHSC.edu