of such a cosmid insert should be a portion of the fragile site. (Microcell transfer of the translocations to fresh rodent backgrounds or isolation of the translocations through chromosome sorting should significantly enhance the ability to detect the translocation junction.) Although it remains possible that the break actually occurred distal to the fragile site, the human portion of the translocation junction would still be closer than 5 cM from the site. Indeed, the genetic distance of this region may be inflated because of unusually frequent recombination (*19*) such that a nearby DNA fragment may be physically closer to the fragile site than the distance of 5 cM suggests. This physical distance would approach the resolution of pulsed field and field inversion gel electrophoresis as well as "jumping" libraries (*20*). Furthermore, such nearby fragments, if not of the fragile site itself, would be immediately useful for linkage analysis in families segregating the fragile X chromosome (*21*).

Several conclusions can be drawn from the data presented. First, it is shown that a fragile site can influence specific chromosome breakage within the dividing cell. Second, this fragile site-mediated breakage can lead to nonrandom chromosome rearrangement involving the fragile site. This finding is of relevance concerning the postulated relationship between certain autosomal fragile sites and the breakpoints of nonrandom chromosome translocations seen in leukemias and lymphomas (*5*). Third, the observation of reduced chromosome fragility at the translocation junctions lends support for the model of the fragile X site as a reiterated DNA sequence. Lastly, the method of identifying cell hybrids that carry chromosome rearrangements of a human fragile site and rodent DNA suggests a means for molecular cloning of fragile site sequences, and may be used for selected autosomal fragile sites where appropriate markers exist.

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Smooth Muscle–Mediated Connective Tissue Remodeling in Pulmonary Hypertension

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Abnormal accumulation of connective tissue in blood vessels contributes to alterations in vascular physiology associated with disease states such as hypertension and atherosclerosis. Elastin synthesis was studied in blood vessels from newborn calves with severe pulmonary hypertension induced by alveolar hypoxia in order to investigate the cellular stimuli that elicit changes in pulmonary arterial connective tissue production. A two- to fourfold increase in elastin production was observed in pulmonary artery tissue and medial smooth muscle cells from hypertensive calves. This stimulation of elastin production was accompanied by a corresponding increase in elastin messenger RNA consistent with regulation at the transcriptional level. Conditioned serum harvested from cultures of pulmonary artery smooth muscle cells isolated from hypertensive animals contained one or more low molecular weight elastogenic factors that stimulated the production of elastin in both fibroblasts and smooth muscle cells and altered the chemotactic responsiveness of fibroblasts to elastin peptides. These results suggest that connective tissue changes in the pulmonary vasculature in response to pulmonary hypertension are orchestrated by the medial smooth muscle cell through the generation of specific differentiation factors that alter both the secretory phenotype and responsive properties of surrounding cells.

PERSISTENT PULMONARY HYPERTENsion in infancy leads to severe lung vascular changes that include marked hypertrophy, extension of vascular smooth muscle to small vessels, adventitial thickening, and increased connective tissue deposition in both the media and adventitia (*1, 2*). In animal models of pulmonary hypertension, increased collagen deposition appears to be important in causing altered mechanical properties of hypertensive vessels (*3, 4*), but other connective tissue components also

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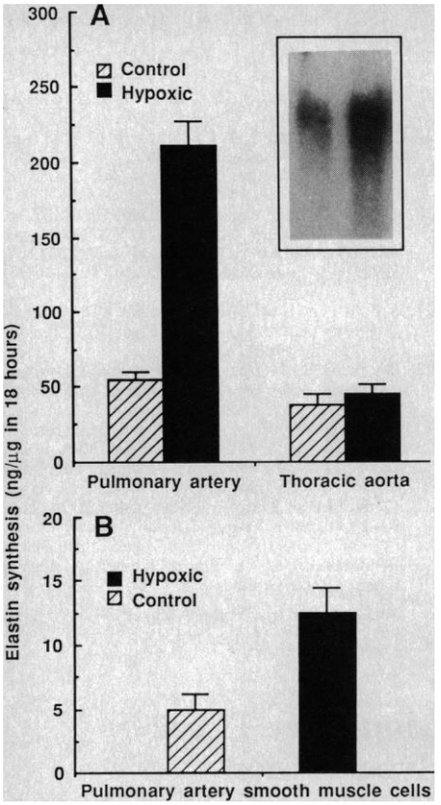

Fig. 1. (**A**) Elastin production and elastin mRNA levels in the lobar pulmonary artery and thoracic aorta of a control and hypoxic calf. Pulmonary artery from the origin of the left middle lobe to the sixth-order branch and a 3-cm segment of the thoracic aorta taken immediately distal to the ductus were minced into approximately 1-mm cubes and incubated overnight at 37°C in high-glucose Dulbecco's modified Eagle's medium supplemented with nonessential amino acids, 30 m*M* Hepes (*p*H 7.4), 10% fetal bovine serum, β-aminopropionitrile (100 μg/ml), and penicillamine (50 μg/ml). Tropoelastin was extracted by homogenizing in acetic acid (*14*) and assayed by competitive enzyme-linked immunosorbent assay (ELISA) (*14*). Values are expressed as nanograms of α-elastin equivalents per microgram of DNA (mean ± SD of triplicate determinations) in 18 hours. (**Inset**) Northern blot analysis of elastin mRNA levels in pulmonary artery. RNA was isolated as described (*15*), and equal loadings (10 μg per lane) of glyoxyl-dimethyl sulfoxide–denatured total RNA were separated on a 1% agarose gel and blotted onto nitrocellulose. Hybridization was with nick-translated elastin probe pSS1 (*16*) and showed a single band at 3.5 kb. Left lane, control animal; right lane, hypoxic animal. (**B**) Elastin production by pulmonary artery medial SMCs from control and hypoxic animals. Confluent first-passage cells, grown from explants of arterial medial tissue, were incubated overnight in culture medium as described above. Tropoelastin in the medium and in a 0.5*N* acetic acid extract of the cell layer was determined by ELISA (*14*). Values are means ± SD of triplicate determinations of culture medium and cell layer combined. Total protein synthesis, as assessed by [^{3}H]leucine incorporation into trichloroacetic acid–insoluble protein, was similar for both control cells and cells from hypoxic animals.

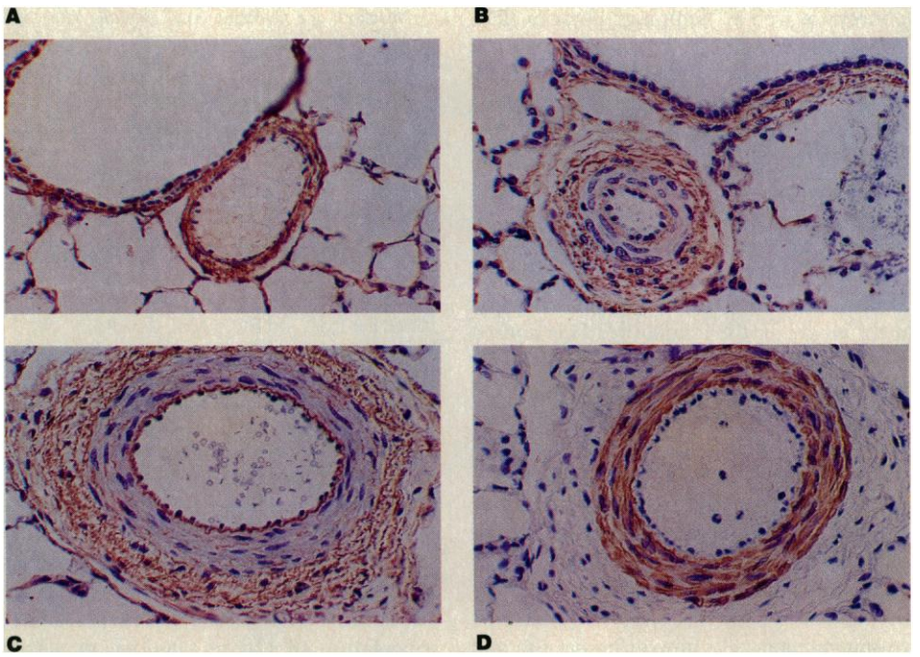

Fig. 2. Immunoperoxidase staining of pulmonary arteries with antibodies to elastin and type IV collagen. Bovine lung tissues were fixed by perfusion through the airways with 10% neutral-buffered formaldehyde solution and embedded in paraffin. For immunohistochemistry, 5-μm sections were prepared as described (*15*) and incubated in a moist chamber for 2 hours at 37°C with affinity-purified rabbit antibody to human type IV collagen (*17*) (10 mg/ml) or mouse monoclonal antibody [immunoglobulin G (IgG)] to bovine α-elastin (*18*) (25 mg/ml). Negative controls consisted of parallel sections incubated with normal mouse or rabbit IgG, respectively. Washed sections were incubated for 20 minutes with affinity-purified biotin-conjugated goat antibody to mouse (BRL; 1:1600 in phosphate-buffered saline containing 1% bovine serum albumin) or goat antibody to rabbit IgG (BRL; 1:800), washed, and incubated for 20 minutes with a 1:400 dilution of streptavidin-horseradish peroxidase (1:10). Complexes were visualized by incubation for 20 minutes with 3,3′-diaminobenzidine (Sigma; 0.5 mg/ml in 50 m*M* tris-HCl, *p*H 7.4) and H_2O_2. Sections were washed, counterstained with Harris hematoxylin, dehydrated, mounted in Permount, and examined by light microscopy. Original magnification, ×125. (**A**) Control animal. (**B**) Hypoxic animal. (**C** and **D**) Slightly larger, more proximal vessels from the same hypoxic animal as (B). (A) and (C) were stained with antibody to elastin and (D) was stained with antibody to type IV collagen. The thickened and hypercellular adventitia in hypertensive vessels contains numerous fibers which stain for elastin (B and C) whereas type IV staining surrounds individual SMCs in the thickened media (D).

increase in this condition. Elastin, the extracellular matrix protein responsible for elastic recoil of arteries, is synthesized during vascular development by smooth muscle cells (SMCs) (*5*). This highly cross-linked protein is localized principally in the medial layer of the vessel, but in hypertensive pulmonary arteries, increased elastin can be observed in the adventitia (*2, 6, 7*), suggesting that adventitial fibroblasts have undergone a phenotypic transition to become elastogenic cells. This observation led us to utilize alterations in elastin synthesis as an assay to investigate the molecular events and cellular signals responsible for differential regulation of connective tissue production in vascular disease.

Pulmonary hypertension in the newborn calf is an attractive model for studying mechanisms underlying alterations in extracellular matrix accumulation associated with vascular abnormalities. The newborn calf, when placed at high altitude, develops hypoxic pulmonary vasoconstriction and severe pulmonary hypertension accompanied by changes in lung vessels that mimic those found in infantile persistent pulmonary hypertension (*7, 8*). Indeed, after 2 weeks at a simulated altitude of 4300 m (440 mmHg), pulmonary arterial pressure is higher than systemic pressure, and the pulmonary arterial vasculature shows medial and adventitial thickening, distal muscularization of those pulmonary arterioles associated with decreased luminal diameter, and an eventual loss of vasoreactivity due in part to increased vascular connective tissue (*4*).

To compare elastin production in vessels of control animals and animals that had been made hypoxic by simulated high altitude, we freed the left middle lobe pulmonary artery from adherent lung tissue through the sixth-order branch. When elastin production was determined in the minced vessel, we found elastin synthesis to be approximately four times higher in the pulmonary artery from

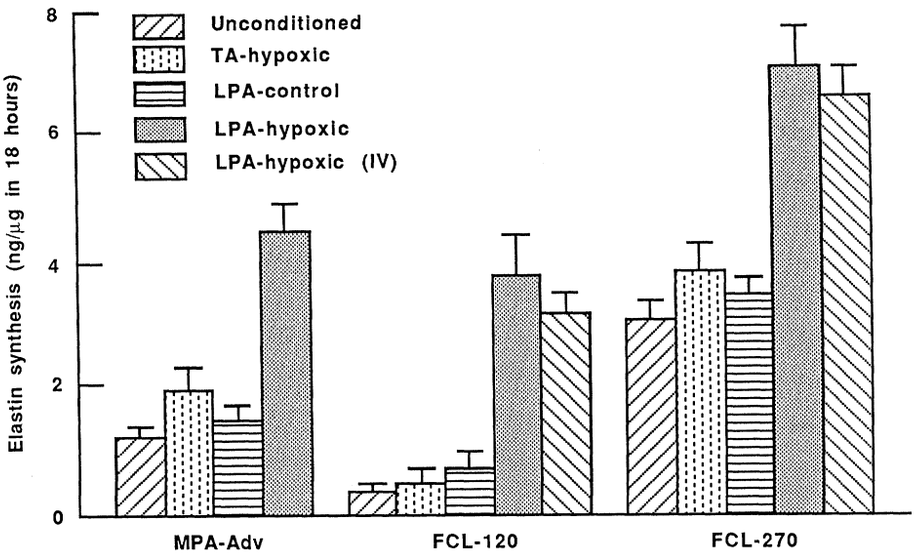

Fig. 3. The effect of smooth muscle–conditioned serum on elastin production by fibroblasts. SMCs from medial tissue explants of thoracic aorta (TA) and lobar pulmonary artery (LPA) from a hypoxic and control calf were seeded into 900-cm^2 roller bottles and maintained in growth medium consisting of Dulbecco's modified Eagle's medium supplemented with 20 m*M* Hepes, nonessential amino acids, and 10% fetal bovine serum. Shortly after the cells reached visual confluency, the growth medium was replaced with 100% calf serum for 18 hours. The conditioned serum was then diluted to 10% with growth medium or was fractionated by Sephadex G-100 chromatography; the fraction containing elastogenic activity (fraction IV) was lyophilized, diluted with Dulbecco's medium to the volume of serum applied to the column, and diluted to 10% with growth medium. Freshly passaged fibroblasts from the adventitial layer of main pulmonary artery of control animals (MPA-Adv) or from the ligamentum nuchae of either 120-day (FCL-120) or 270-day (FCL-270) fetal calves were then cultured in growth medium supplemented with conditioned serum or fraction IV. Unconditioned calf serum diluted in growth medium or fractionated over Sephadex G-100 served as control. After confluency was reached, elastin production was determined for each cell type by competitive ELISA (*14*). Values are means and standard deviation of triplicate determinations.

hypoxic animals than that from controls (Fig. 1A). Northern blot analysis of RNA extracted from the tissue and hybridized with a DNA probe for elastin demonstrated an increase in elastin-specific messenger RNA (mRNA), thereby correlating the increase in elastin synthesis with increased mRNA levels. In contrast to the differences observed in the pulmonary artery, elastin production and elastin mRNA levels in the thoracic aorta from control and hypoxic animals were not significantly different (Fig. 1A). That the thoracic aorta was unaffected in the animals made hypoxic by simulated high altitude was not surprising since blood pressure in the systemic circulation is not increased at high altitude (*7*).

The results described above demonstrate a dramatic increase in the rate of elastin synthesis in the hypertensive pulmonary artery. To determine whether these changes resulted from stable phenotypic alterations in vascular cells, we compared elastin production in cultured pulmonary artery medial SMCs from control and hypoxic animals. Cultured SMCs from hypoxic animals produced more elastin than control SMCs (Fig. 1B). The increase in elastin production appears to reflect a stable phenotype since relative differences between control cells and cells from hypoxic animals persisted in vitro through at least three culture passages.

Histochemical studies of smaller elastic and muscular arteries in the hypertensive rat lung have revealed extensive remodeling in the adventitia as well as in the media (*6, 7*). In particular, a marked accumulation of elastin-rich matrix external to the media indicates increased elastin synthesis by the adventitial cells. A similar increase in elastin-rich matrix in hypertensive bovine lung was confirmed by immunoperoxidase staining with antibodies to elastin. Elastin staining was observed in all layers of the artery but was markedly increased in the adventitia (Fig. 2). Staining with monospecific antibodies to type IV collagen, which stains the basal lamina of SMCs, further demonstrated the marked increase in connective tissue external to the thickened media.

Little is known about how the adventitial cell might be modulated to express the elastin phenotype. Our previous finding that SMCs can influence elastin synthesis by cultured endothelial cells (*9*) suggested the possibility that the elastin phenotype is influenced in the diseased vessel by specific factors released locally by activated SMCs. Early-passage medial cells in roller bottles were exposed overnight to 100% calf serum to test for the presence of an elastogenic differentiation factor released by the SMCs from hypoxic animals. Samples of the conditioned serum were then tested for effects on elastin synthesis in fibroblasts isolated from

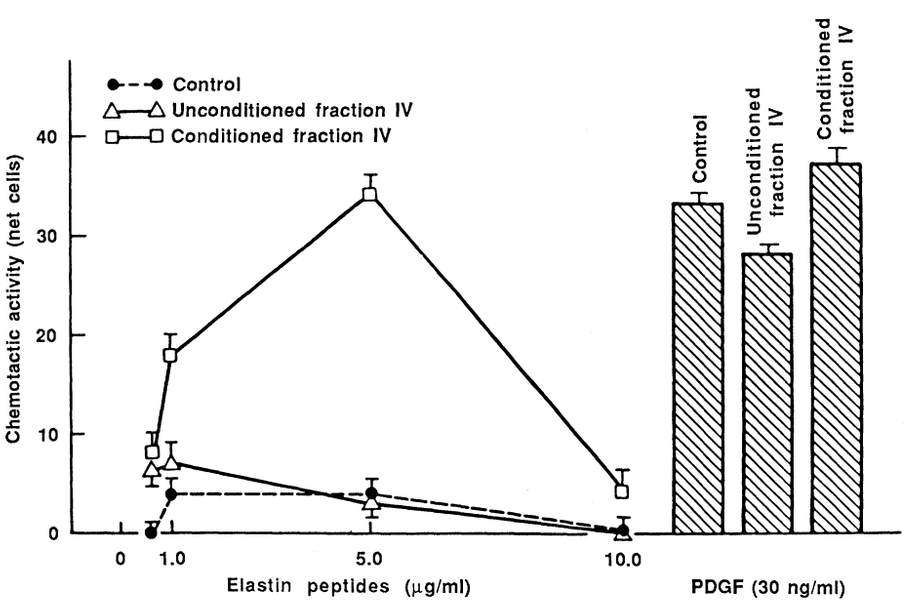

Fig. 4. Induction of chemotactic responsiveness to elastin peptides. Ligamentum nuchae fibroblasts from a 95-day bovine fetus were cultured in growth medium alone (control), in growth medium containing the Sephadex G-100 fraction of smooth muscle–conditioned serum that contained elastogenic activity (conditioned IV) (see legend to Fig. 3), or the corresponding Sephadex G-100 fraction of unconditioned serum (unconditioned IV). Chemotactic responsiveness to elastin peptides and to 1 n*M* PDGF (30 ng/ml) was assayed by a double micropore membrane system in modified Boyden chambers as described (*19*). Values are expressed as net cells per high power field and are means ± SE for 15 determinations.

adventitia of normal pulmonary artery. Production of elastin by the adventitial cell was enhanced by serum conditioned by SMCs from the pulmonary artery of hypoxic animals, but not by unconditioned serum or by conditioned serum from aortic cells or from pulmonary vascular cells of control animals (Fig. 3). The elastogenic stimulatory effect seems to be a response of the SMC to high serum levels since activity could not be detected in culture medium supplemented with only 10% (v/v) calf serum. The amount of stimulatory activity present in conditioned serum showed variation with different serum lots, and consistently greater activity was obtained with calf serum than with fetal bovine serum.

To determine whether the smooth muscle–derived elastogenic factor (SMEF) acts at a transcriptional or post-transcriptional level, we studied the effects of conditioned serum on the differentiation of fetal ligamentum nuchae fibroblasts. In these cells, the elastin phenotype is actively expressed in late gestation (older than 180 days), whereas in early gestation the phenotype is inactive but inducible (*10*). Thus, ligament fibroblasts early and late in gestation can be used in a convenient well-characterized bioassay to determine whether factors induce new elastin gene expression or simply stimulate elastin synthesis in cells that are already actively expressing the elastin phenotype.

When differentiated (late gestation) ligament fibroblasts were cultured in medium containing serum conditioned by SMCs from the pulmonary artery of hypoxic animals, there was a two- to fourfold stimulation of elastin production compared to cells grown in unconditioned medium or in serum conditioned by SMCs from normal pulmonary artery or thoracic aorta (Fig. 3). Total protein synthesis, as determined by [^{3}H]leucine incorporation into trichloroacetic acid–insoluble protein, was unaltered by the conditioned medium. Significantly, undifferentiated (early gestation) cells cultured in the presence of conditioned serum from the SMCs of hypoxic animals took on phenotypic and morphological features of differentiated cells, as evidenced by the onset of elastin production (Fig. 3) and a stellate morphology. These results demonstrate that SMEF functions both as an enhancer of elastin synthesis and as a differentiation factor, inducing expression of the elastin phenotype in responsive cells.

The inductive property of smooth muscle–conditioned serum was substantiated by investigating its effects on another differentiation-related property of ligament cells—namely, chemotaxis in response to elastin-derived peptides. We previously showed that the appearance of chemotactic responsiveness to elastin peptides in fetal ligament fibroblasts parallels the onset of elastin synthesis and that both of these phenomena are sensitive markers of differentiation (*11*). Figure 4 shows that undifferentiated ligament cells grown in medium supplemented with column fraction IV from SMC-conditioned serum (see below) acquired chemotactic responsiveness to elastin peptides characteristic of differentiated cells. No chemotactic response was observed when cells were grown in fraction IV from unconditioned serum, even though the cells remained fully responsive to the fibroblast chemoattractant platelet-derived growth factor (PDGF). These results not only provide evidence for an altered secretory phenotype in response to SMEF, but also suggest that cell surface receptors are changing as well. Cell surface modulation could have important physiological consequences by altering receptor-mediated responses such as cell movement or the ability of the cell to respond metabolically to external stimuli. Thus, both the secretory and responsive properties of the cell may be changed after exposure to SMEF.

The biochemical characteristics of the elastogenic activity released by hypertensive pulmonary artery SMCs are not yet known. Indeed, it is not clear whether SMCs produce SMEF directly or whether they process or alter serum or plasma factors to produce SMEF. It is also uncertain whether the activity involves more than one factor. When SMEF-containing serum was fractionated by Sephadex G-100 chromatography, elastin-stimulating activity was found as a broad peak eluting near the column total volume (fraction IV), corresponding to a molecular weight range of less than 15,000 (based on the exclusion properties of globular proteins). Cells grown in medium containing this fraction showed changes identical to those described above for cells grown in the presence of unfractionated conditioned serum (Figs. 3 and 4).

In summary, these results suggest that the pulmonary artery SMC plays a critical role in the pathogenesis of vascular changes of pulmonary hypertension by modifying the phenotype of surrounding cells in the vessel wall. Our data support a model in which exposure of medial SMCs to plasma factors after vessel wall injury results in a phenotypically altered cell that generates SMEF. SMEF, in turn, stimulates or induces elastin synthesis and perhaps synthesis of other connective tissue macromolecules. Also, because SMEF or an associated activity affects chemotactic responsiveness of vascular cells, smooth muscle factors could modulate responsiveness to external stimuli generally. If the SMC assumes an autocrine-like function in the hypertensive state after vessel injury, these findings could have significance for other vascular diseases such as atherosclerosis, in which there is evidence that the SMC (*12*) is responsible for many of the cellular and functional features of the atherosclerotic lesion (*13*). The finding that the pulmonary artery SMC in pulmonary hypertension generates a factor (or factors) that affects connective tissue synthesis by other cells in the vessel wall provides a direction for further research into molecular and cellular mechanisms of connective tissue regulation in diseased vessels.

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