American Journal of Pathology, Vol. 141, No. 6, December 1992

Ultrastructural Analysis of Contractile Cell Development in Lung Microvessels in Hyperoxic Pulmonary Hypertension

Fibroblasts and Intermediate Cells Selectively Reorganize Nonmuscular Segments

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The current study traces the development of contractile cells in the nonmuscular segments of rat lung microvessels in hyperoxic pulmonary hypertension. New intimal cells first develop into a well-defined layer beneath the endothelium and internal to an elastic lamina. Ultrastructurally, these cells are found to be 1) fibroblasts recruited to the vessel wall from the interstitium and 2) intermediate cells, a population of preexisting vascular cells (structurally between a smooth muscle cell and a pericyte). Early in hyperoxia (days 3 through 7), interstitial fibroblasts migrate and align around the smallest vessels in which an elastic lamina is either absent or fragmentary. These cells then are incorporated into the vessel wall by tropoelastin secretion and the formation of an elastic lamina along their abluminal margin. After day 7, the new mural fibroblasts acquire the features of contractile cells, namely a basal lamina, extensive microfilaments, and dense bodies. In other vessels, as early as day 3 of hyperoxia, intermediate cells within the vessel intima begin to acquire the additional filaments and dense bodies of contractile cells. As hyperoxia continues, each cell pathway gives rise to vessels with distinct intimal or medial layers of contractile cells. In this way, thick-walled 'newly muscularized' vessel segments form adjacent to the capillary bed. (Am J Pathol 1992, 141:1491–1505)

In hyperoxic pulmonary hypertension, wall thickening restricts the lumen of the myriads of small vessels that enter and drain the capillary bed. These findings have been established in the rat, by hemodynamic measurements *in vivo*, and, by the morphometric analysis of arteries and veins identified by barium-gelatin injection.[1–4] Typically, a new layer of medial muscle develops in the smallest of these vessels, increasing the proportion with a complete or partially muscular wall at the expense of ones nonmuscular. The aim of this study is to establish for these vessels the cellular basis of wall thickening and muscle development.

In small precapillary and postcapillary segments of normal rat lung, intimal cells are found beneath the endothelium and internal to an elastic lamina. In most segments they are relatively sparse, and include smooth muscle cells, intermediate cells, and pericytes.[5–7] In location and morphology, the intermediate cell lies between the smooth muscle cell and pericyte, having filaments but no dense bodies, and an enclosing basal lamina.[6] Reconstruction of an intra-acinar axial arterial pathway has shown a gradation in the arrangement of these cell types, smooth muscle cells giving way to intermediate cells and intermediate cells to pericytes.[7] Along this pathway, smooth muscle cells extend distally into the region of the vessel wall associated with second-generation alveolar ducts, and intermediate cells into the precapillary region that lies within the alveolar wall. Pericytes line lung capillaries.[8]

In the current study, the development of intimal and medial cells is followed in vessel segments associated with alveolar ducts and walls, especially the smallest alveolar wall segments, where intermediate cells but not smooth muscle cells are normally found. The distribution of intimal, medial, and adventitial cells is analyzed by light and transmission microscopy, and by a montage of pho-

Supported by NIH grant RO1 HL34552.
Accepted for publication June 15, 1992.
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tographic prints. The first technique provides a large sample of vessel profiles from which to evaluate the distribution and sequence of wall changes; the latter classifies cell phenotype.

Quantitative analysis confirms that, in response to hyperoxia, hypertrophy and hyperplasia of intimal, medial, and adventitial cells thickens the vessel wall. Typically, a new intimal subendothelial cell layer develops in virtually all nonmuscular vessels and in the nonmuscular region of half of the partially muscular vessel population. Existing intermediate cells within the vessel wall, and fibroblasts recruited to the vessel wall from the interstitium, are found to form this, by developing into single or multiple layers of contractile cells beneath the endothelium. On occasion, both cell types reorganize the wall of the same vessel.

Materials and Methods

Male viral antibody-free Sprague-Dawley rats (Charles River Breeding Laboratories, Portage, MI) were used in two experiments (A and B) that were carried out on separate occasions. All animals received food (Purina rat chow 5008) and water *ad libitum*.

Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW publication No. [NIH] 78-23, revised 1978).

At the start of each experiment, all animals were of similar age and weight. They were randomly assigned to a group; a total of 45 untreated rats were controls and 57 rats breathed high oxygen.[1,2] In experiment A, vascular cells were analyzed in rats breathing 87% O_2 for 1, 2, 4, 7, 15, or 28 days (n = 5 at each time) and in control rats at similar times (n = 3 at each time). In experiment B, rats breathed 87% O_2 for 1, 3, 4, 7, 9, 12, 14, 21, or 28 days (n = 3 at each time), or were controls (n = 3 at each time). Tissue from experiment B, as well as additional tissue from experiment A, was examined by transmission microscopy.

Tissue Preparation

All animals were injected with sodium pentobarbital (30 mg/100 g body weight, intraperitoneally); the treated rats continued to breathe high oxygen until they were deeply anesthetized. The lungs were excised and the pulmonary arteries and airways uniformly distended with 2% glutaraldehyde in 0.1 mol/l cacodylate buffer (at 100 and 23 cm H_2O pressure, respectively), the arteries being filled slightly ahead of the airways. After fixation (30 to 40 minutes), tissue blocks of 1 to 2 mm^2 were selected from the base of the left lung and right cardiac and diaphragmatic lobes (i.e., 1 to 2 mm from the pleural edge). The blocks were further fixed (30 minutes), washed well in 0.1 mol/l cacodylate buffer, postfixed in 1% OSO_4 (60 minutes), processed, and embedded in Epon-araldite. Thick sections (1 μ) were stained with toluidine blue (1% in 1% borax); thin sections (700 Å) were stained with 1% uranyl acetate in 100% methanol and Reynolds lead citrate, and examined at 60 kV.

Quantitative Analysis of Vascular Cells

In experiment A, all alveolar wall and alveolar duct vessels in 1-μm sections of the tissue blocks from each control and each hyperoxic rat (i.e., five blocks from each of three lung regions for each animal) were examined qualitatively. Cell size and number were analyzed to assess the contribution of each cell type to wall thickening, (microscope field size diameter, 0.42 mm). Tissue blocks were chosen at random from four control (two at the start and two at the end of the study) and 15 hyperoxic rats (three each at 2, 4, 7, 15, and 28 days). Vessels in which cells were analyzed were characterized as muscular (by a medial layer of cells between an external and internal elastic lamina), as partially muscular (by a media present in only part of the wall), or as nonmuscular (by the absence of a media). The external diameter (ED) and medial thickness (MT) of each vessel was measured, the percent medial thickness (%MT) calculated,[2] and their location (as alveolar wall or duct) was noted. Cell diameter was measured across the widest zone of the nucleus, and cell concentration was expressed per unit length of vessel circumference. Only nucleated cell profiles were included.

Intimal cells abutting the vessel lumen were classed as endothelial; intimal cells beneath the endothelium were classed not by cell type but as a group, and termed subendothelial. At different time points in hyperoxia, transmission microscopy demonstrated that a variety of cell types formed this layer (i.e., intermediate cells, fibroblasts, smooth muscle cells—and each of these cells in transition—as well as pericytes), but cell phenotype is not identified in 1-μm sections viewed by light microscopy. Cells between an internal and external elastic lamina, however, were classed as smooth muscle cells, because these were the cells always identified at this location ultrastructurally. Similarly, cells aligned to the vessel contour and abluminal to a single or external elastic lamina were classed as adventitial fibroblasts, as were additional cells (in the hyperoxic lung) that were obviously fibro-

blasts but closely aligned around vessels in which a lamina was absent.

In analyzing the above data, levels of significance were calculated by the unpaired *t*-test, with Bonferroni's correction factor adopted to adjust the significance level. A result was taken to be statistically significant if the *P* value was less than 0.01. Morphometric findings for the control groups were not significantly different and so they were combined. Change in the distribution of vessels in the hyperoxic lung (by size and wall structure) was assessed by chi-square analysis.[1]

Ultrastructural Analysis of Vascular Cells

In 1-μm sections of each tissue block, vessels were identified that demonstrated the important wall changes at each time point in the hyperoxic lung. These same vessels were then viewed by electron microscopy and photographed (×1500 and ×9450 to ×12,060). The walls of some were photographed serially at high magnification—a total of 50 hyperoxic vessels and 30 normal ones. Contact prints allowed quick assembly of a vessel montage from which to assess wall changes, and select additional regions for photography (×18,900). This approach allowed a detailed ultrastructural analysis to be made of a large number of vessels.

Ultrastructurally, cells were identified and classed by their morphology (shape and distribution of organelles), basal lamina, and location in relation to other cells and wall components. The cells found in the walls of normal alveolar wall and duct vessels are described here as a frame of reference. The endothelial cell lies adluminal to a basal lamina and to a single or internal elastic lamina. Rough and smooth endoplasmic reticulum, mitochondria, and a Golgi complex typically are distributed throughout the cell, and micropinocytotic vesicles usually found along the adluminal cell margin. The *pericyte* is an elongated cell with long thin processes arising from its pole to envelop the vessel wall. It shares the basal lamina of the endothelial cell (which splits to surround both cells)[8] and lies internal to a single elastic lamina. Processes along the adluminal cell margin may extend to interdigitate with the cytoplasm of the endothelial cell. Organelles are sparse, few intracytoplasmic filaments are found, and the focal densities typical of a smooth muscle cell are absent. The intermediate cell is named for its location and morphology (midway between a smooth muscle cell and pericyte). Like the smooth muscle cell, it is cigar-shaped and has relatively few organelles, few or many microfilaments (4 to 6 nm diameter, i.e., actin) and a separate basal lamina. Like the pericyte, it lacks dense bodies and lies internal to a single elastic lamina. Single cells, or two overlapping cells, are usually found. Typically, the medial smooth muscle cell is cigar-shaped, and the cytoplasm is packed with microfilaments (4 to 6 nm diameter). Focal densities run parallel to the filaments. Few mitochondria, only sparse endoplasmic reticulum, and an undeveloped Golgi complex are found adjacent to the poles of the nucleus. Micropinocytotic vesicles are numerous, and subplasmalemmal densities are present. The cell is surrounded by a distinct basal lamina. Hemidesmosomes usually are present at the polar ends of adjacent cells. Single or overlapping cells are found between an internal and external elastic lamina. The *adventitial fibroblast* is a spindle-shaped cell with extended processes. Endoplasmic reticulum and ribosomes are usually abundant and the Golgi complex well-developed. This cell lacks a basal lamina. It closely follows the contour of the vessel, and is abluminal, and in direct apposition to, a single or external elastic lamina. Single cells are usually present; and they are relatively infrequent. Collagen fibers usually are associated with only the abluminal cell margin. The interstitial fibroblast resembles the adventitial cell morphologically, but is more irregular in shape and found in the thick region of the alveolar–capillary membrane. Cell processes frequently extend into the thin region separating the epithelial basal lamina from that of the endothelial cell. Collagen fibers may surround this cell or be associated with only a single margin.

Results

Body Weight

Between days 4 and 7 of hyperoxia, most rats weighed less than at the start of exposure (experiment A, Table 1). Between days 15 and 28, most had gained weight, but their absolute weight (expressed as the mean value for the group) remained below that of age-matched controls. All of the control rats steadily gained weight. Similar results were obtained for the other groups of rats included in experiment B (data not given).

Quantitative Analysis of Vascular Cells

Examples of alveolar duct and wall vessels with subendothelial cells in the hyperoxic lung, as well as an alveolar wall vessel with a newly developed medial layer, are illustrated in Figure 1a through d.

Cell Hypertrophy and Hyperplasia

Intimal cells, both endothelial and subendothelial, and medial smooth muscle cells, were hypertrophied by day 7 of hyperoxia, adventitial fibroblasts by day 4 (Figure 2a

Table 1. *Experiment A—Effect of Hyperoxia (87% O_2) on Body Weight (mean ± SEM)*

	Animal group	Starting weight (g)	Final weight (g)
Group 1	Control (3)	207 + 5	Day 1 211 + 6
Group 1	87% O_2(5)	210 + 4	Day 1 213 + 4
Group 2	Control (3)	209 + 11	Day 2 219 + 13
Group 2	87% O_2(5)	207 + 2	Day 2 204 + 5
Group 3	Control (3)	202 + 7	Day 4 230 + 9
Group 3	87% O_2(5)	222 + 5	Day 4 190 + 5*
Group 4	Control (3)	212 + 1	Day 4 264 + 6
Group 4	87% O_2(5)	220 + 4	Day 7 264 + 6
Group 5	Control (3)	221 + 10	Day 15 318 + 6
Group 5	87% O_2(5)	213 + 2	Day 15 226 + 7*
Group 6	Control (3)	210 + 3	Day 28 358 + 16
Group 6	87% O_2(5)	211 + 1	Day 28 223 + 8*

Numbers in parenthesis = number of rats; *$P \leq 0.01$ compared with age-matched control value. Starting weight = day 0.

and b, $P \leq 0.001$ for each cell type *versus* control value). Vascular cell hypertrophy then persisted.

In most vessels the concentration of vascular cells was low at day 2 of hyperoxia; because the count reflected nucleated profiles, cell edema and swelling were the likely basis of this reduction. In small vessels (≤50 μ ED), the endothelial cell number then remained low, and in larger ones (≥50 ≤ 100 μm ED), it regained the normal value only at day 28 (Figure 3a, $P \leq 0.01$ for each vessel category *versus* control value). The concentration of subendothelial cells increased first in the small vessels, i.e., at day 4 (Figure 3a, $P \leq 0.01$). In the large vessels it had increased by day 7 ($P \leq 0.01$ for each vessel category). In both vessel groups, the concentration of these cells had doubled by day 28 ($P \leq 0.01$ for each vessel category).

The concentration of medial smooth muscle cells did not significantly increase in the small or large vessels until day 28 (Figure 3b, when $P \leq 0.01$ for each vessel category *versus* control value). The concentration of adventitial fibroblasts increased around the small vessels by day 2 (Figure 3b, $P \leq 0.01$) and around the large vessels by day 15 ($P \leq 0.01$). In both, it then remained high (day 28 $P \leq 0.01$ for each vessel category).

Distribution of Vessels with Intimal Subendothelial Cells or Medial Smooth Muscle Cells

As hyperoxia progressed, more nonmuscular than partially muscular vessels developed a subendothelial cell layer (Figure 4a and b). Chi-square analysis of the vessel population confirmed that as quickly as the subendothelial cell layer developed in some vessels, a media formed in others (i.e., by day 4). Vessels with a complete or partial media increased at the expense of vessels without (p_x^2 0.001 on days 4 through 28). Early in hyperoxia, most of these were duct vessels.

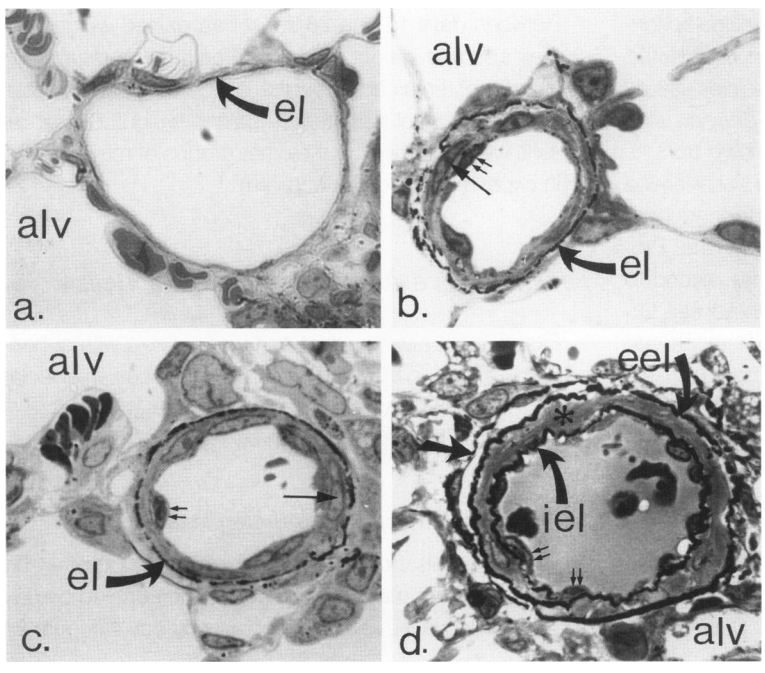


Figure 1. *Alveolar vessels (1-μm toluidine blue sections): endothelial cells (double arrows), subendothelial cells (single arrow), alveolus (alv), elastic lamina (el):* **a**: *Alveolar wall vessel (ED 30 μm, ×1156) in normal lung showing fine elastin deposits forming a fragmentary elastic lamina; the vessel is extremely thin-walled with only endothelial cell processes present.* **b**: *Thick-walled alveolar wall vessel (ED 25 μm, ×1083), after 28 days 87% O_2, with nucleated endothelial cells and subendothelial cells internal to a well-defined single elastic lamina:* **c**: *Thick-walled alveolar duct vessel (ED 26.5 μm, ×1238), as for* (**b**): **d**: *Thick-walled alveolar wall vessel (ED 44 μm, ×1800) with medial smooth muscle cells (*) between a well-defined internal (iel) and external elastic lamina (eel). Elastin deposits form an additional and fragmentary lamina in part of the wall (arrow). In this and in subsequent figures the original print magnification is given.*

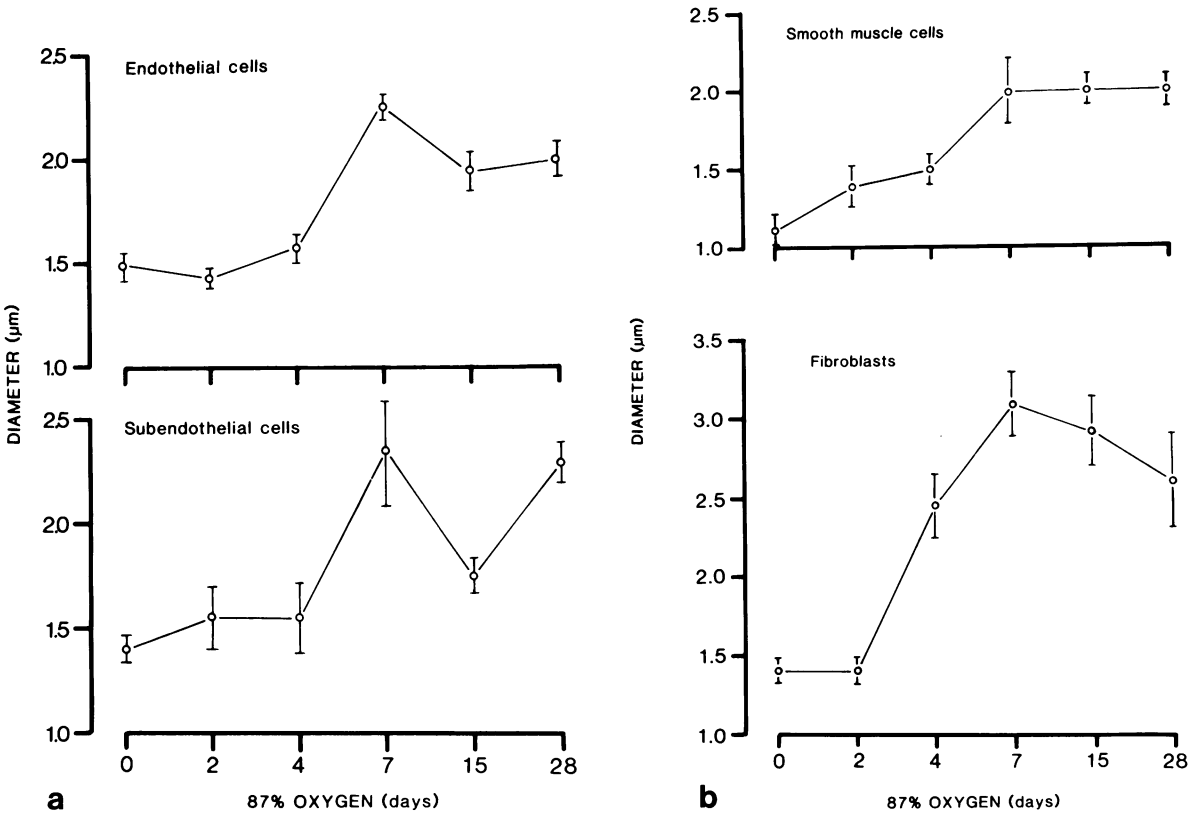


Figure 2. *Vascular cell hypertrophy in alveolar wall and duct vessels (≤100 μm ED) in normal lung (day 0) and after 87% O_2 (days 2, 4, 7, 15, and 28). Mean cell diameter ± SEM;* (a) *endothelial and subendothelial cells;* (b) *smooth muscle cells and fibroblasts (see text).*

Vessel Narrowing

In the vessels analyzed, wall thickening was accompanied by an increase in the relative number of small vessels at the expense of larger ones (p_x^2 0.001 on days 4 through 28), indicating either a change in wall distensibility—as by contracture—or the inclusion of smaller vessels, i.e., ones that developed in a lamina *de novo* in hyperoxia (see below).

Ultrastructural Analysis of Vascular Cells

Wall reorganization in the smallest nonmuscular alveolar wall vessels (i.e., ≤50 μm ED) of the hyperoxic lung is illustrated (Figures 5 through 13); the pattern was similar in alveolar duct vessels, although these vessels mainly were reorganized by intermediate cells, not fibroblasts (see below).

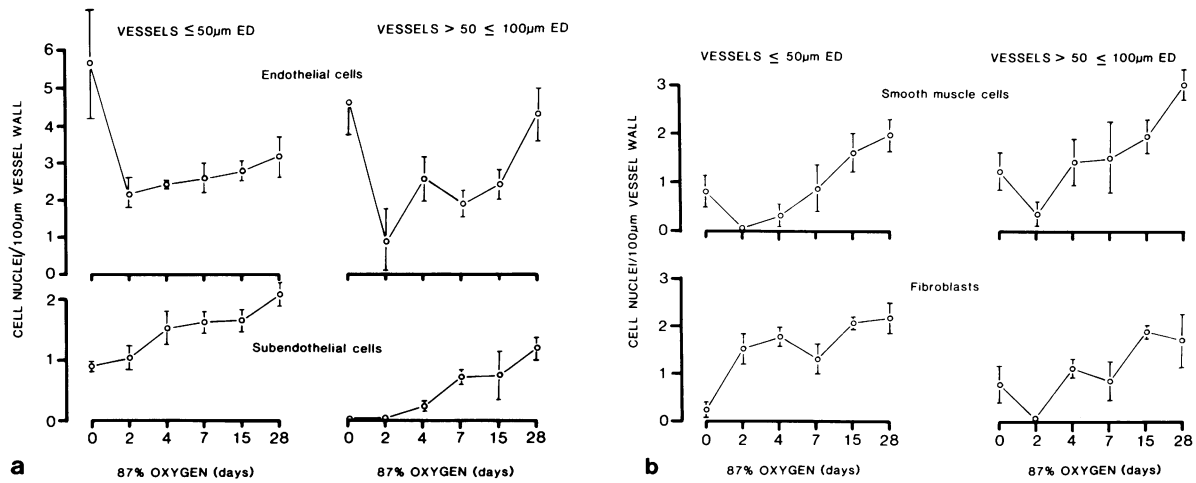


Figure 3. *Vascular cell hyperplasia in alveolar wall and duct vessels grouped by size, i.e., ≤50 μm and >50 ≤ 100 μm ED, in normal lung (day 0) and after 87% O_2 (days 2, 4, 7, 15, and 28). Mean cell number ± SEM per unit length of vessel wall);* (a) *endothelial and subendothelial cells;* (b) *smooth muscle cells and fibroblasts (see text).*

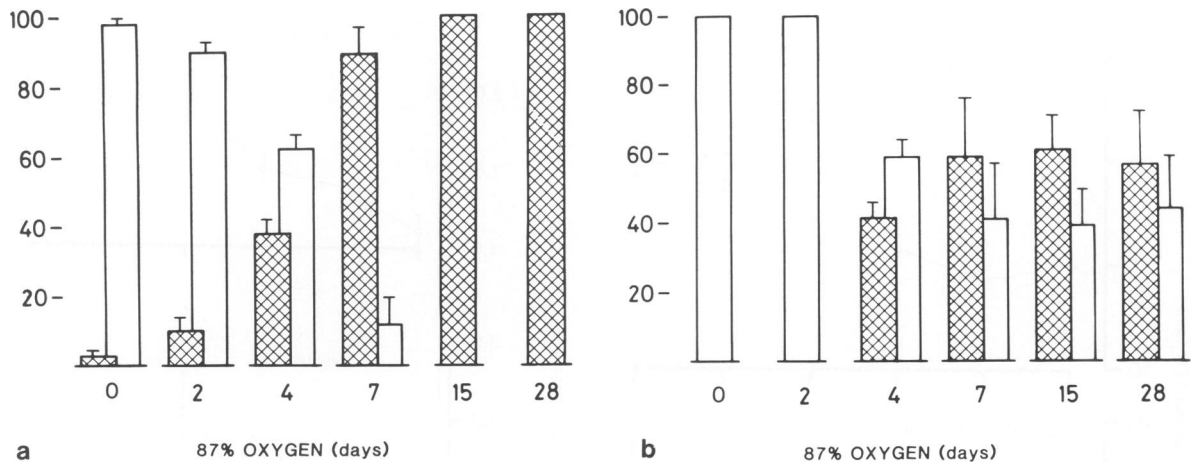


Figure 4. *Percentage (mean ± SEM) of alveolar wall and duct vessels (≤100 μm ED) with subendothelial cells (hatched bars) and without these cells (open bars), in normal lung (day 0) and after 87% O_2 (days 2, 4, 7, 15, and 28).* **a**: *Nonmuscular vessels: in normal lung, vessels with subendothelial cells form <3% of the nonmuscular population: Between day 2 and day 15 of hyperoxia this proportion increases to virtually 100%.* **b**: *Partially muscular vessels: in normal lung, and at day 2 of hyperoxia, none of these vessels have subendothelial cells. By day 4 of hyperoxia about 40% have subendothelial cells and by day 7 56%. This proportion of the vessel population does not then change. Within partially muscular vessels, subendothelial cells were found only in the nonmuscular region of the wall, and no muscular vessels had subendothelial cells.*

Normal Structure of the Nonmuscular Segment

In normal rat lung, alveolar wall vessels consisted of endothelial cell processes bound by basal lamina (Figure 5). A thin lamina of fibrillar elastin with amorphous deposits was present in most vessels > 25 μm ED: In some <25 μm ED, and in many < 20 μm ED, a lamina was absent, these vessels thus being larger than capillaries by size but with a similar wall structure.

In most of the larger vessels (> 25 ≤ 100 μm ED), and in some small ones (≤25 μm ED), intermediate cells formed a subendothelial cell layer (Figure 5). Pericytes were seen only in some of the smallest vessels and in capillaries. Alveolar wall vessels as large as 100 μm and as small as 15 μm in diameter were seen to give rise to a capillary. The wall structure of these juxtacapillary vessels was typical for size.

Reorganization of the Nonmuscular Segment in Hyperoxia

The sequence of vessel wall reorganization by 1) fibroblasts and 2) intermediate cells is described. This was based on the analysis of tissue at the level of the cell, vessel, and vessel population.

Fibroblast Pathway

Early in hyperoxia (days 2 through 4), proteinaceous fluid expanded the interstitium around many alveolar wall vessels > 15 < 25 μ ED (Figure 6a), especially those in which an elastic lamina was fragmentary, or absent. This loosened the interstitium around vessels in the thickest region of the alveolar-capillary membrane, and by collecting between the endothelial and epithelial basal lamina, created a new perivascular space around vessels in thinner regions. Interstitial fibroblast processes often extended through this space to approximate the endothelial basal lamina (Figure 6a). In some vessels, the processes of intermediate cells were present between the approaching fibroblast and the endothelium (Figure 6b). Inflammatory cells also were seen in this perivascular region.

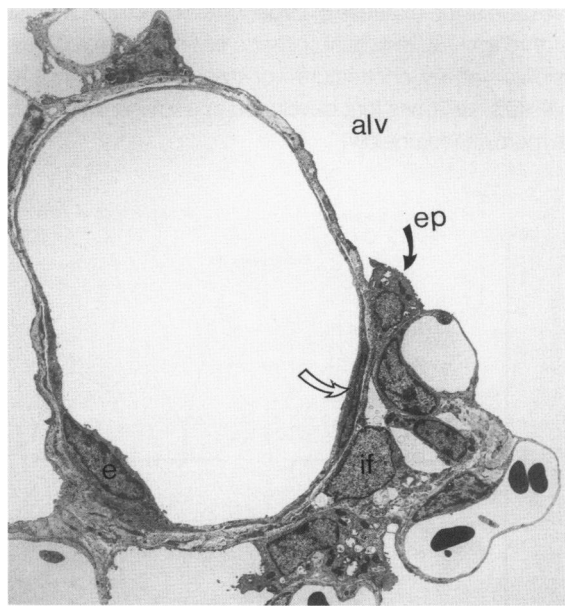
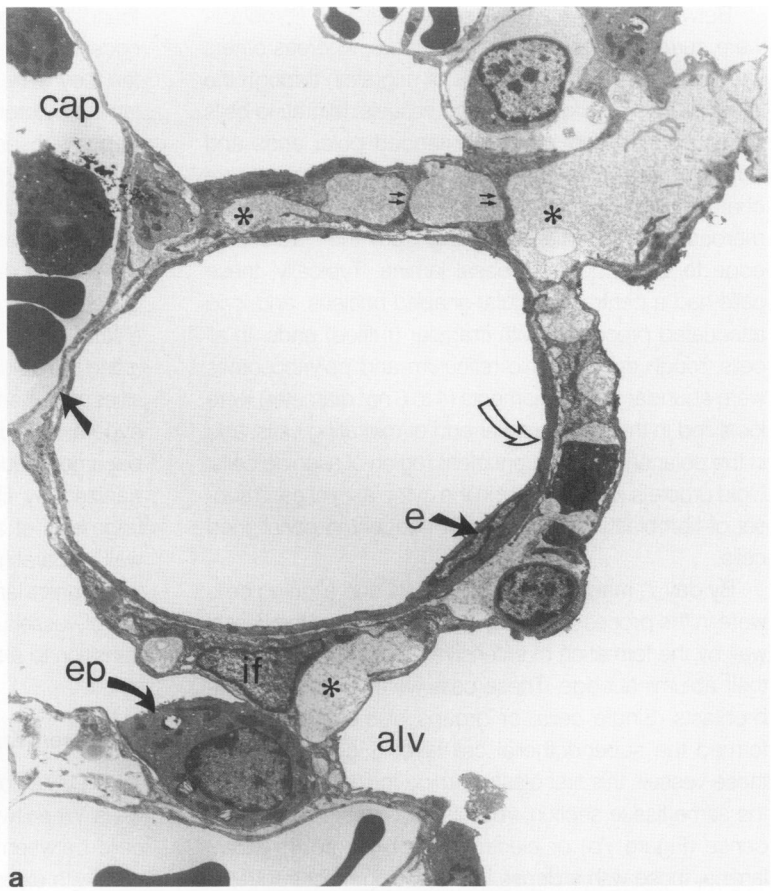


Figure 5. *Nonmuscular alveolar wall vessel (ED 25 μm, ×3954) in normal lung: endothelial cell (e), interstitial fibroblast (if), epithelial type 2 cell (ep). At higher magnification, an additional cell (at open arrow) was classed as an intermediate cell (not shown).*



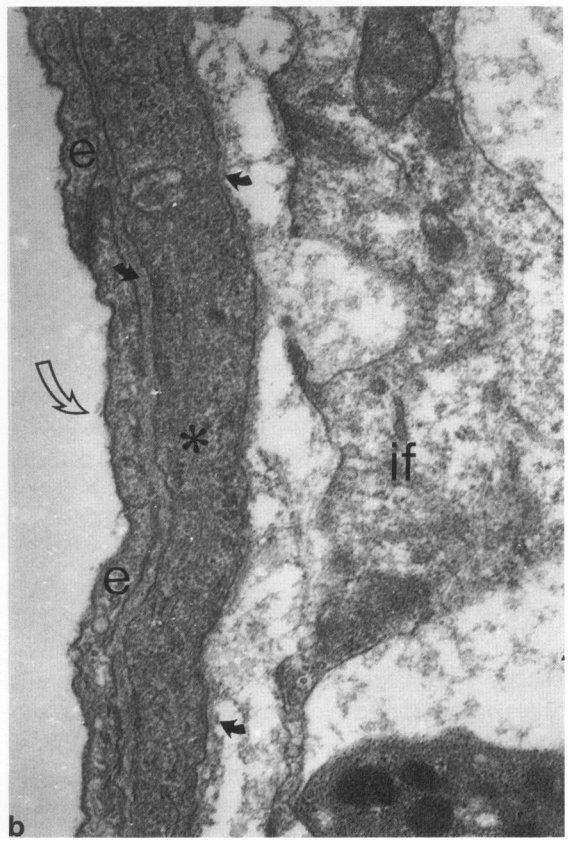


Figure 6. *Alveolar wall vessel (ED 18.6 μm, ×4500 and ×56,700) after 3 days 87% O_2: interstitial fibroblast (if), epithelial type 2 cell (ep), alveolus (alv), capillary (cap), endothelial cell (e).* a: *Proteinaceous fluid widens and creates an interstitial space around the vessel (*). The processes of an interstitial fibroblast extend around the left side of the vessel, those of another fibroblast cross the interstitial space (double arrows). In part of the wall (at open arrow), another cell process lies between a fibroblast process and the endothelium* (b). b: *Region of wall (at open arrow) showing the process of a cell classed as an intermediate cell (*) separated from endothelium and from the approaching processes of an interstitial fibroblast by electron-dense basal lamina and extracellular matrix components (small arrows).*

Between days 4 and 7, some interstitial fibroblasts were surrounded by collagen deposits, whereas others were at a stage in the process of migration through the interstitium, or in aligning around vessels. Migrating cells were characterized by their extended polar ends and filopodia, and a trailing cell body; those aligning were characterized by their location, orientation, and by numerous lamellapodia extending from their adluminal edge to the endothelial basal lamina. Typically, these cells had a central triangular-shaped nucleus, and long attenuated processes with irregular (ruffled) ends. In all cells, rough endoplasmic reticulum and polyribosomes were abundant; microfilaments (4 to 6 nm diameter) were localized in the leading polar end of migrating cells, and in the polar ends and subnuclear region of aligning cells. Lipid droplets were present in the cytoplasm of each subset of fibroblasts but were most frequent in nonaligned cells.

By day 7, many aligned fibroblasts and aligning cells were in the process of being incorporated into the vessel wall by the formation of extensive elastin deposits along their abluminal edge. These cells were termed mural fibroblasts. Single cells, or groups of these cells, now formed the subendothelial cell layer (Figures 7, 8). In these vessels this first elastic lamina formed *de novo*. In the same tissue section, vessels had either an electron-dense (Figure 7b) or electron-lucent (Figure 8) elastic lamina, those with a dense lamina being most frequent.

Between days 7 and 28, the walls of these vessels became more organized as the mural fibroblasts developed a basal lamina and contractile organelles, i.e., extensive microfilaments and dense bodies. Numerous micropinocytotic vesicles were present, especially along the abluminal margin (Figure 9). Further deposits thickened the elastic lamina, or appeared as isolated fragments within the vessel wall. As hyperoxia progressed, more and more of these now contractile cells developed subplasmalemmal densities (indicating tethering or attachment devices) along their adluminal and abluminal margins; and hemidesmosomes formed. In some vessels, the development of a contractile cell phenotype was associated with tropoelastin secretion along the adluminal cell margin and the formation of fragments of a second or internal elastic lamina, which separated the cell from the endothelial basal lamina.

Intermediate Cell Pathway

By day 3 of hyperoxia, in vessels of all sizes, but especially in the larger ones (i.e., $\geqslant 50 \leqslant 100$ μm ED), single or overlapping intermediate cells formed the subendothelial layer (Figure 10a, b). Typically, cigar-shaped cells, with a similar nucleus, and microfilaments, these cells were enclosed by basal lamina. They were usually found adluminal to a single preexisting elastic lamina; on occasion, no elastic lamina was evident (Figure 10). Often they were separated from the endothelial cell basal lamina by proteinaceous fluid and electron-dense matrix deposits, especially in the first days of hyperoxia.

Between days 3 and 7, intermediate cells were still evident in some vessels; in others, cigar-shaped cells with a well-developed basal lamina, many microfilaments, and dense bodies indicated cells that had acquired a contractile cell phenotype. Microfilaments were distributed throughout these cells but were most dense along the adluminal cell margin. Subplasmalemmal densities and hemidesmosomes were rarely seen early in hyperoxia (<day 4) but later (days 14 through 28), these became frequent. In some vessels, as in the ones reorganized by fibroblasts, tropoelastin secretion formed fragments of a second or internal lamina in the vessel wall, separating the new contractile cell from the endothelial basal lamina. Between day 7 and day 28, the number of vessels with these cells, i.e., (intermediate cells in transition to a smooth muscle cell) increased.

Vessel Wall Structure at Day 28

At Day 28 of hyperoxia, most vessels had reorganized walls. When two double elastic laminae were present, the cells between were typical smooth muscle cells, (i.e., cells with extensive filaments, dense bodies, basal lamina, subplasmalemmal densities, and hemidesmosomes). Other vessels still had intimal cells that varied in the expression of their contractile organelles (Figures 11, 12, 14). Cells within a single vessel, however, were notably uniform. In other vessels, cells with extensive open arrays of endoplasmic reticulum and ribosomes, and incomplete basal lamina, indicated synthetic activity.

In the vessels in which contractile cells now predominated, it was difficult to discern the cell pathway responsible for wall reorganization. Certain characteristics in the appearance of a vessel indicated one or other pathway. Triangular-shaped contractile cells, forming single or multiple layers, and a marked elastic lamina, were found in both the intima or media of some vessels. In a single profile, the long axis of these cells was not always parallel to the vessel lumen. Acellular regions persisted in some of these vessels, but in most the cells were now well organized, albeit into eccentric layers. These features indicated contractile cells that had developed from fibroblasts (Figures 11, 12, 13). Elongated cells aligned in a regular fashion parallel to the lumen, also found in the intima and media of vessels, indicated the intermediate cell pathway (Figures 1b, 1c, 14).

Pericytes formed the subendothelial cell layer in some of the smallest vessels of the hyperoxic lung (Figure 15) and were obvious in capillary walls.

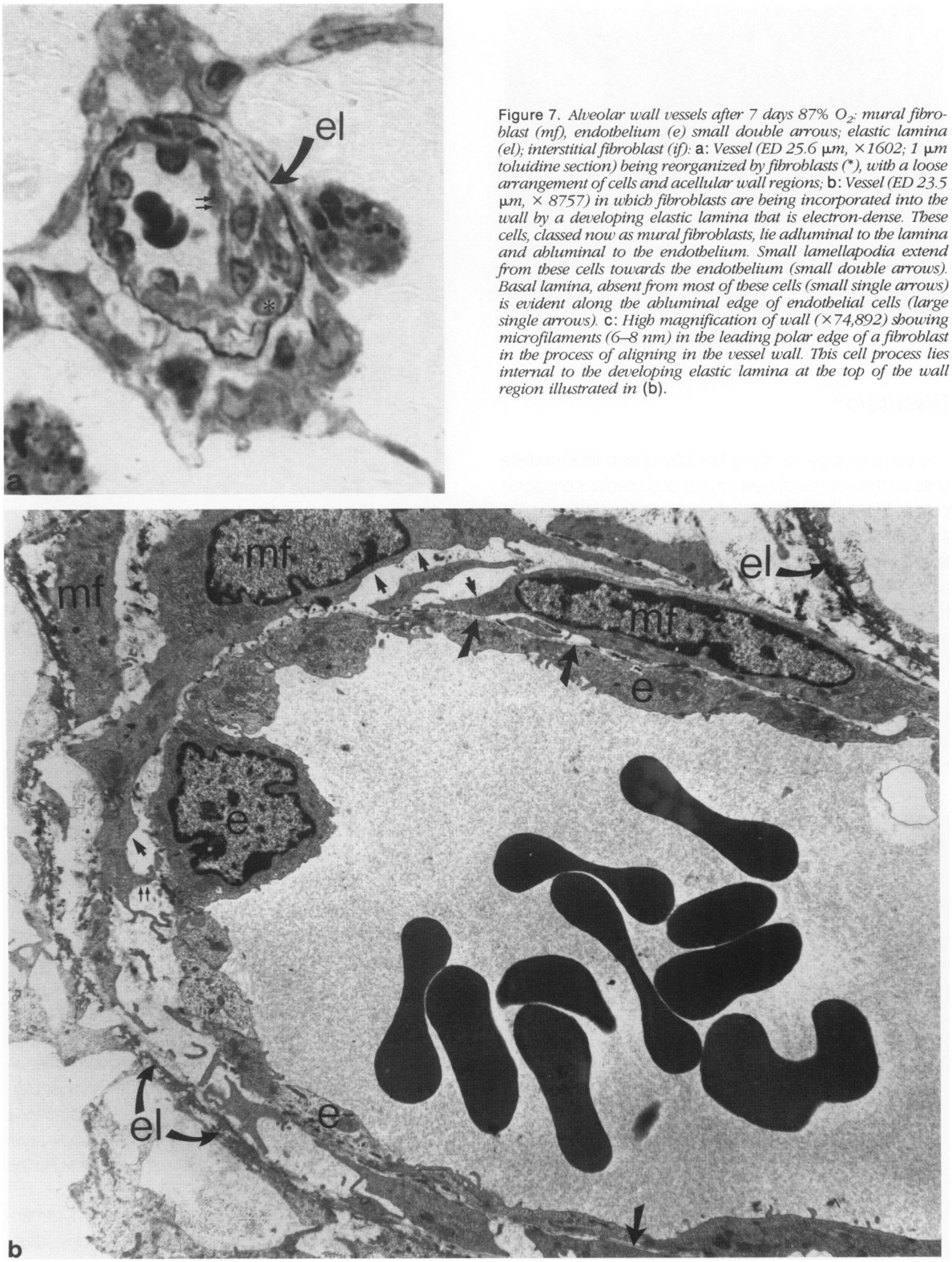


Figure 7. *Alveolar wall vessels after 7 days 87% O_2: mural fibroblast (mf), endothelium (e) small double arrows; elastic lamina (el); interstitial fibroblast (if):* a: *Vessel (ED 25.6 μm, ×1602; 1 μm toluidine section) being reorganized by fibroblasts (*), with a loose arrangement of cells and acellular wall regions;* b: *Vessel (ED 23.5 μm, × 8757) in which fibroblasts are being incorporated into the wall by a developing elastic lamina that is electron-dense. These cells, classed now as mural fibroblasts, lie adluminal to the lamina and abluminal to the endothelium. Small lamellapodia extend from these cells towards the endothelium (small double arrows). Basal lamina, absent from most of these cells (small single arrows) is evident along the abluminal edge of endothelial cells (large single arrows).* c: *High magnification of wall (×74,892) showing microfilaments (6–8 nm) in the leading polar edge of a fibroblast in the process of aligning in the vessel wall. This cell process lies internal to the developing elastic lamina at the top of the wall region illustrated in* (b).

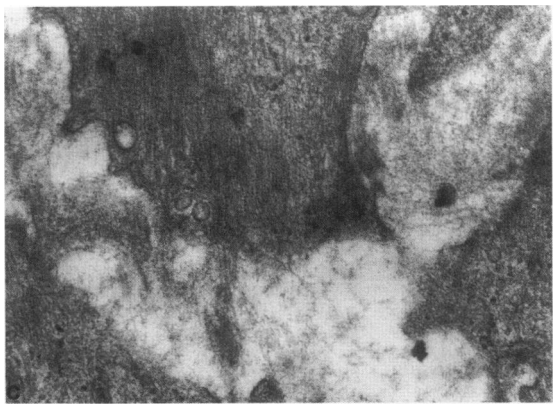


Figure 7. *(Continued).*

Discussion

The current study identifies fibroblasts and intermediate cells as the source of new intimal and medial contractile cells in lung microvessels in hyperoxia. In some vessels, the wall is reorganized by the recruitment of new cells from the interstitial space, in others by the development of a preexisting vascular cell population, and in yet others by both cell pathways. This selective pattern of wall reorganization in the nonmuscular segments of lung microvessels is described here for the first time.

In normal rat lung, thick-walled muscular vessels are found only at the entrance to the acinus (approximately 50% are muscular and 50% partially muscular); the distal intraacinar vessels are extremely thin-walled and have little or no muscle coat. The aim of this study was to establish the cell pathway leading to muscle development in these segments, and, typically, this implies the formation of a new layer of medial cells. In the current study, using day 28 of hyperoxia as an example, the relative percentage of muscular, partially muscular, and nonmuscular alveolar wall vessels was 18%, 20%, and 62%, respectively. Although only 38% of these vessels thus develop medial smooth muscle cells, most of the remaining ones have an additional layer of intimal cells. At this stage of hyperoxia, because most of these are contractile, almost all of the alveolar wall vessels are 'newly muscularized.' Early in hyperoxia (<day 7), however, the presence of a thick intima does not necessarily indicate a new layer of contractile cells because in many of these vessels the cells are still mural fibroblasts.

In a previous study of hypoxic pulmonary hypertension,[9] septal fibroblasts were reported to quickly transform to a mature smooth muscle cell within a media, but it was not shown how this was accomplished. The findings of the current study indicate that such cells are recruited from the interstitium to the vessel wall by stages—

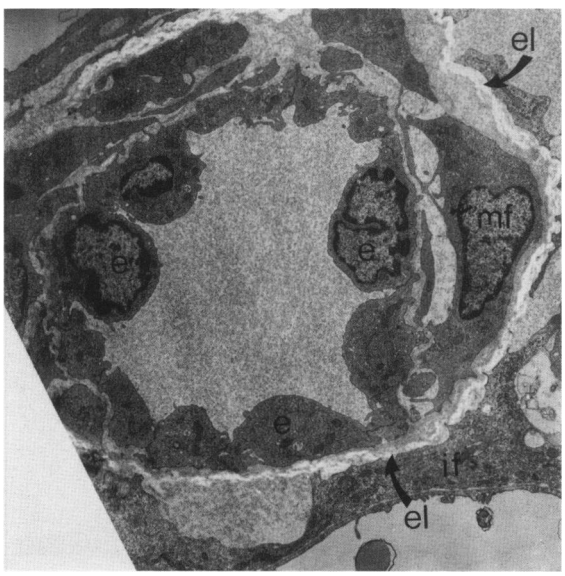


Figure 8. *Alveolar wall vessel (ED 20 μm, ×6570) after 7 days 87% O_2 with mural fibroblasts being incorporated into the wall by a developing elastic lamina that is electron-lucent. Interstitial fibroblasts remain abluminal to the developing lamina.*

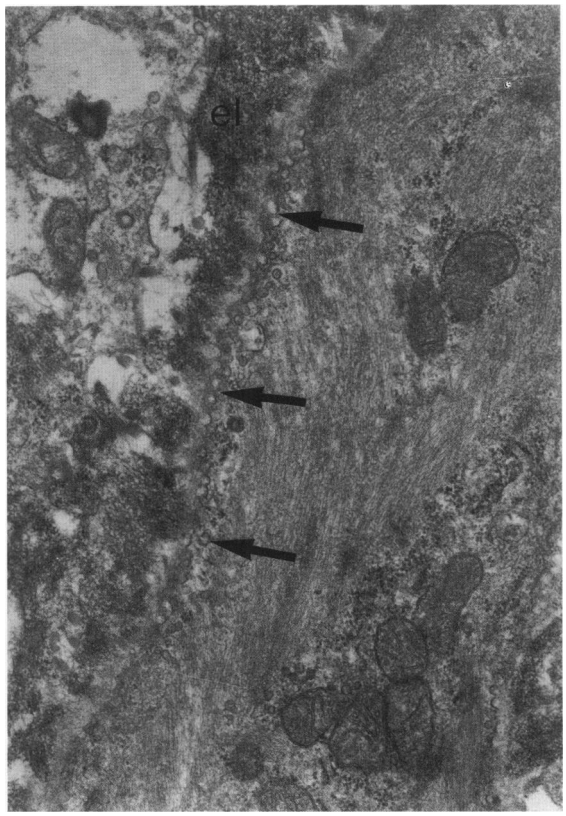


Figure 9. *Alveolar wall vessel (ED 23 μm, ×30,856) after 28 days 87% O_2: numerous vesicles are present along the abluminal edge (open arrows) of a cell process that is abluminal to endothelium and adluminal to the newly forming elastic lamina (el). Microfilaments (6–8 nm) are widely distributed throughout the cell.*

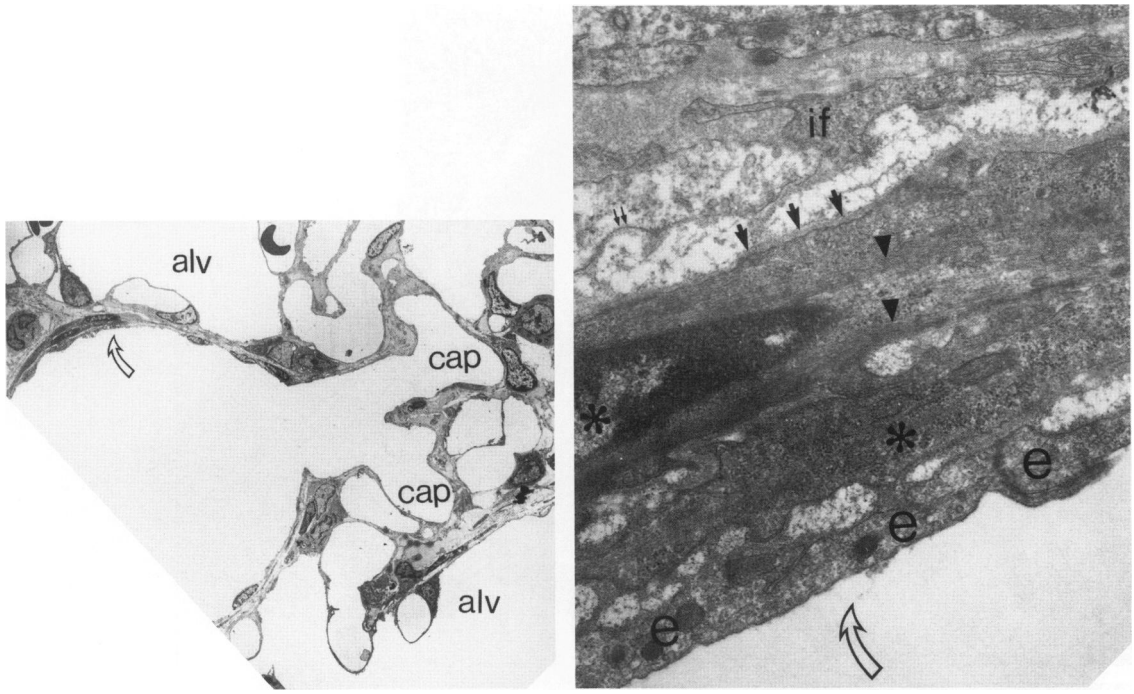


Figure 10. *Alveolar wall vessel with subendothelial cells after 3 days 87% O_2 (ED 34 μm, ×3000 and ×28,350):* **Left:** *Juxtacapillary vessel with additional cells and cell processes (at open arrow) abluminal to endothelium. An elastic lamina is not seen in this region of the wall at this time.* **Right:** *Region of wall (at open arrow) showing the additional cells and cell processes to be those of intermediate cells (*); with extensive filaments (arrowheads), endothelial cell (e), electron-dense basal lamina and extracellular matrix components (small arrows), interstitial fibroblast processes (double arrows).*

1) migration, 2) alignment, and 3) incorporation—each stage being associated with phenotypic modulation in terms of the expression and distribution of actin filaments and tropoelastin secretion. Formation of an elastic lamina is the essential stage in the shift from a fibroblast to a vascular cell, and only then, after incorporation into the wall, is the morphologic expression of a contractile cell phenotype acquired.

In this model, as in other studies of acute and fatal exposures to high oxygen, membrane injury and accumulation of protein-rich fluid occurs in the first days of injury.[10–12] This disturbance in interstitial homeostasis identifies subsets of cells, separating migrating cells from those synthesizing collagen, each perhaps by their response to specific factors in the fluid-filled space. Inflammatory cells, such as the alveolar macrophage, release mediators that stimulate each activity, but the response is determined by the fibroblast.[13] *In vivo,* as *in vitro,*[14] it is likely that migration toward the endothelial cell basal lamina is in response to a chemotactic agent released by the endothelial cell. The structure of migrating cells described here resembles that of those *in vitro*—elongate with a leading pseudopodium and trailing cell process, and with delicate filopodial extensions along the leading cell edge.[15]

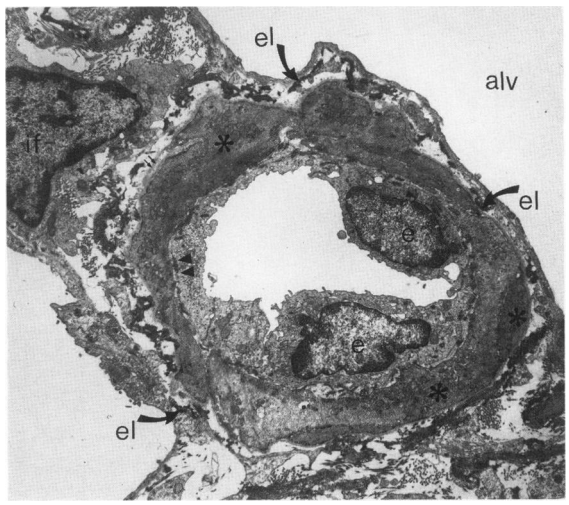


Figure 11. *Alveolar wall vessel with contractile cells forming a subendothelial cell layer, after 28 days 87% O_2 (ED 15.5 μm, ×8750): Subendothelial cell processes (*) lie adluminal to a well-defined electron-dense elastic lamina (el) and abluminal to endothelial cells (e). Basal lamina is evident along the abluminal cell edge (small arrows), and focal elastin deposits adluminally (single arrowheads). The newly developed cells are triangular with the features of a contractile cell; microfilaments are heavily distributed throughout the cell process at the top, less in the process in the lower region of the wall and, at high magnification, dense bodies are seen. Interstitial fibroblast (if), alveolus (alv).*

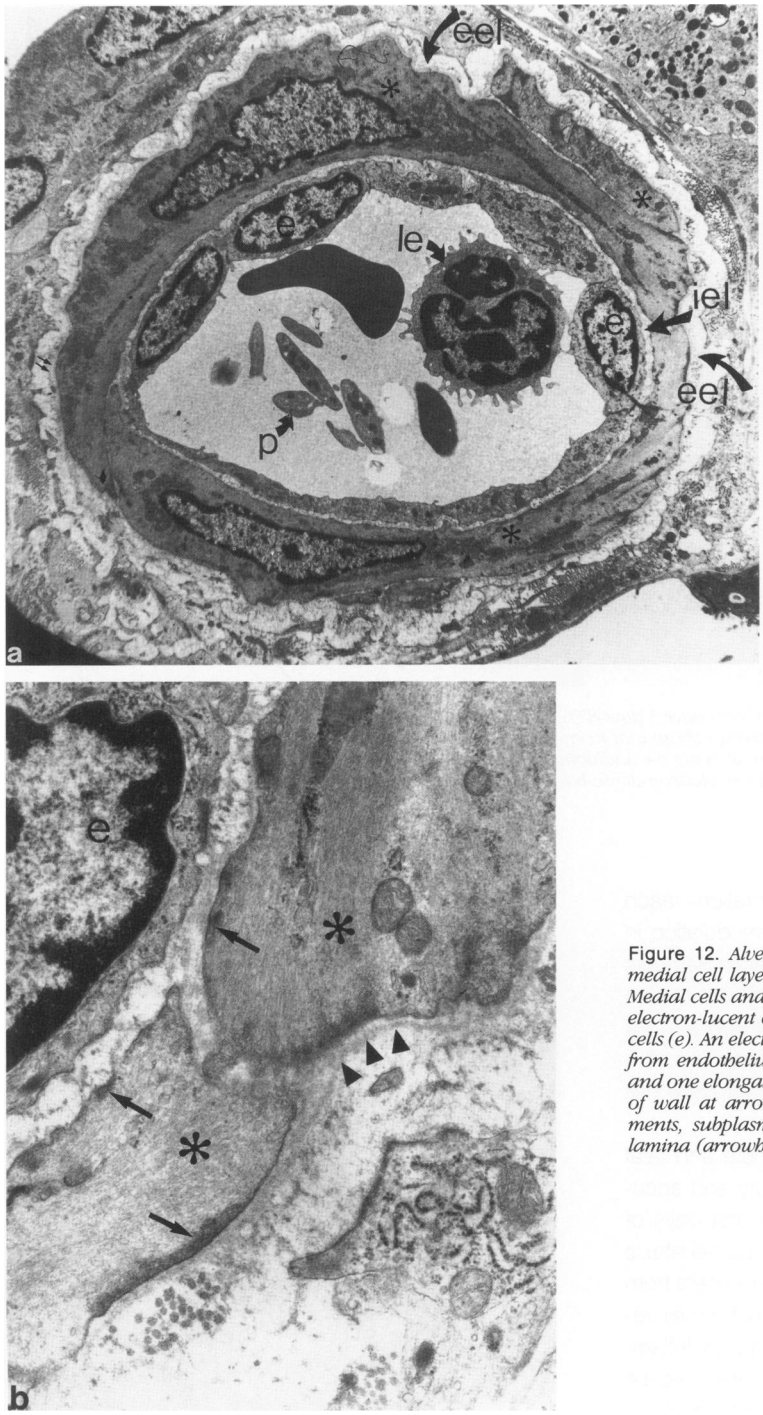


Figure 12. *Alveolar wall vessel with contractile cells forming a medial cell layer, after 28 days 87% O_2 (ED 20 μm, ×8757).* a: *Medial cells and cell processes (*) lie adluminal to a thick external electron-lucent elastic lamina (eel) and abluminal to endothelial cells (e). An electron-lucent internal lamina (iel) separates the cells from endothelium. The cells also are triangular, with one blunt and one elongated polar end. Platelet (p), leukocyte (le).* b: *Region of wall at arrow indicating iel (×28, 673), showing microfilaments, subplasmalemmal densities (arrows) and distinct basal lamina (arrowheads).*

Cell alignment completes the stage of migration, bringing the fibroblast into close apposition with the endothelial cell basal lamina, and orientating it around the vessel lumen. The persistence of gaps in the vessel wall indicates cells that have failed to fully align, or cells that are aligning in a different plane. Our previous data show that fibroblasts aligned around lung microvessels are the most actively proliferating cells in the hyperoxic lung, and that cells present in the wall as a subendothelial layer, i.e., intimal to an elastic lamina, show relatively little proliferative activity.[16] The fibroblast pathway thus provides a significant number of new cells for incorporation into the vessel wall during its reorganization in pulmonary hypertension.

During wall reorganization, the mural fibroblast is clearly identified from the aligning and interstitial fibro-

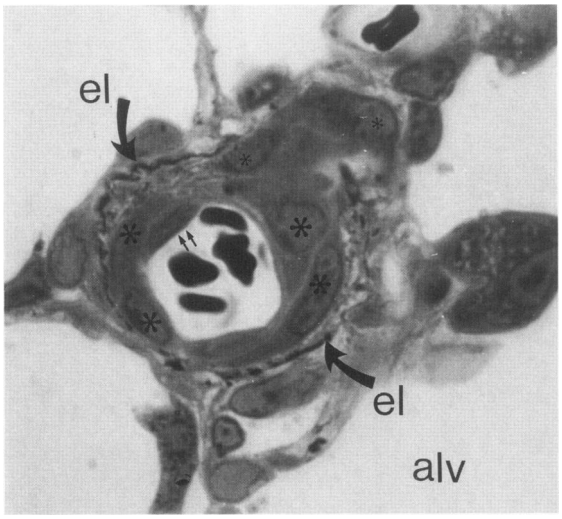


Figure 13. *Alveolar wall vessel after 28 days 87% O_2. Vessel (ED 28 μm, ×1800; 1 μm toluidine blue section) with an incomplete electron-dense elastic lamina surrounding an eccentric collection of cells (*), some of which are contiguous with the interstitial space; endothelial cells (double arrows).*

blast by its location in the vessel intima, internal to a developing elastic lamina. It differs morphologically from the intermediate cell, and from the intermediate cell in transition, by its irregular shape and orientation, presence of lamellipodia, polar distribution of microfilaments, lack of dense bodies, polyribosomes, dilated endoplasmic reticulum, and lack of basal lamina. As it develops contractile organelles, it approaches the morphologic criteria of the fibroblast found in granulation tissue.[17] These cells, termed myofibroblasts, are characterized by numerous parallel arrays of microfilaments and dense bodies, and by a dense bundle of microfilaments similar to the stress fibers of cultured cells.[17–19] The fibroblast in transition to a vascular cell lacks a similar dense bundle of microfilaments. Lacking smooth muscle myosin and a true basal lamina, the myofibroblast has been considered closer to a fibroblast than a smooth muscle cell,[20] but by the distribution of intermediate filament proteins, a range of myofibroblast phenotypes is now recognized.[21] Although the expression of α-smooth muscle actin indicates a shift toward a contractile cell phenotype, desmin is also an important indicator. Further studies are needed to identify these filament proteins in the newly developing intimal and medial cells of the hyperoxic lung.

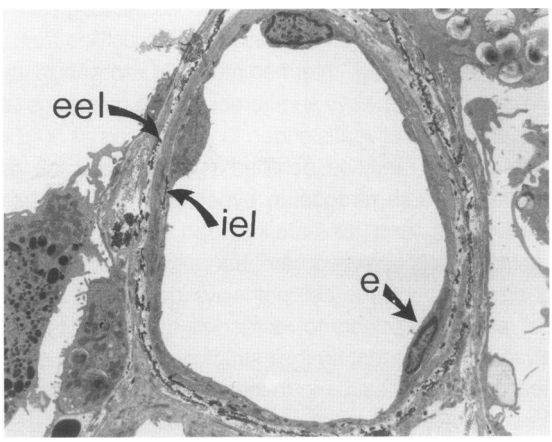


Figure 14. *Alveolar wall vessel with contractile cells forming a subendothelial cell layer, adluminal to the external elastic lamina after 28 days 87% O_2 (ED 24 μm, ×4320): endothelial cell (e), external elastic lamina (eel). Elastin deposits form a fragmentary internal lamina in the vessel wall (iel).*

As a normal intimal cell, the intermediate cell does not need to migrate and align, but rapidly develops a large number of filaments and dense bodies, expressing a contractile cell phenotype more quickly than the transforming fibroblast. Very early in hyperoxia, these cells are easily identified by their location, regular orientation, and by their microfilament density. First recognized in the nonmuscular region of the partially muscular segment of normal rat lung,[6] in hypoxia, they were shown to be the source of new smooth muscle cells in this region while more distally; i.e., in the nonmuscular segment, pericytes were considered likely to fulfill a similar function.[22] The current study shows that the intermediate cell pathway is responsible for wall reorganization in some of the distal segments in the hyperoxic lung. This finding does not agree with that of a previous study of wall remodeling in hypoxic pulmonary hypertension[9] in which intermediate cells were considered unimportant as a source of new smooth muscle cells.

Accepted as present in lung capillaries,[8] it is still unclear whether pericytes commonly extend into the distal nonmuscular vessel segments. They are increasingly considered a contractile cell type[23] and, as such, seem likely to play a role in wall reorganization in the hypertensive lung. Early in hyperoxia, the presence of fluid and wall disruption made it impossible to confirm that cells ever shared the endothelial basal lamina. Frequently, around capillaries and small vessels, the plasmalemma of cells likely to be pericytes was disrupted and cell organelles scattered in the interstitial space. Only later were pericytes clearly seen to thicken the walls (and so narrow the lumen) of vessels that bordered capillaries in size. In this model of pulmonary hypertension, therefore, the pericyte represents a significant cell in terms of lumen restriction, but its role as the source of new smooth muscle cells remains unclear.

Transmission microscopy shows that it is the many small precapillary and postcapillary vessels without an elastic lamina that are reorganized by fibroblasts. A fragmentary lamina located outside the plane of the thin sections examined by transmission microscopy, might explain its absence in these vessel profiles. Despite their structural resemblance to capillaries, these segments are larger in diameter, and are marked by extensive wall re-

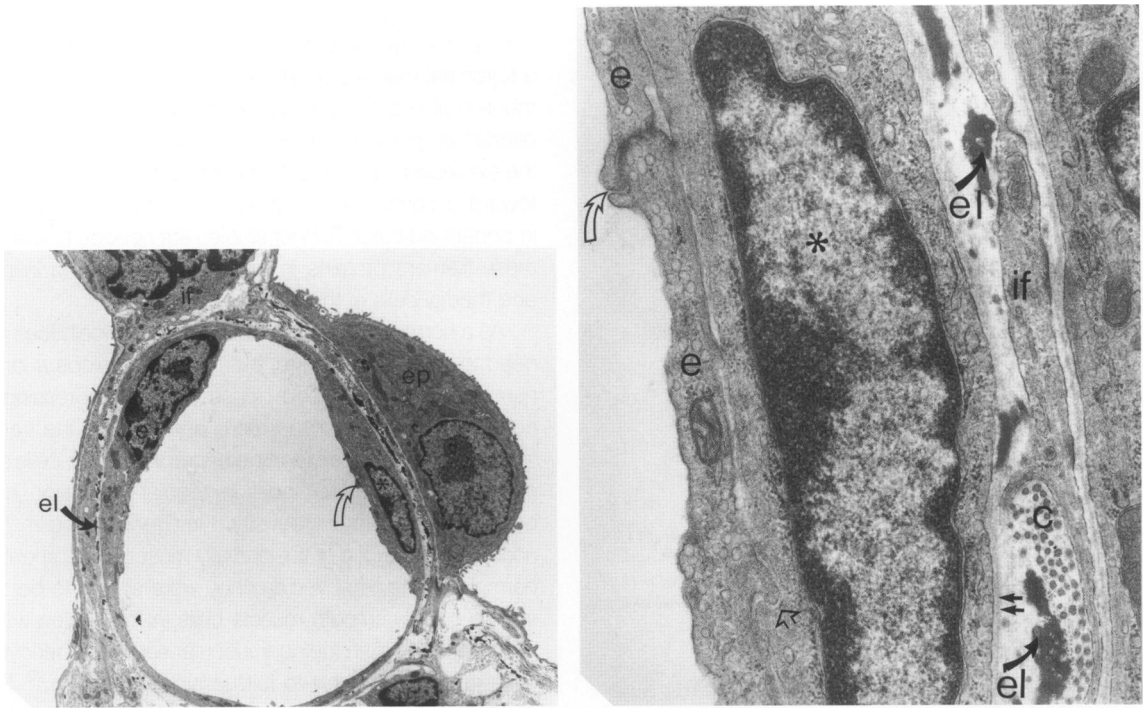


Figure 15. *Alveolar wall vessel with a pericyte after 28 days 87% O_2 (ED 14 μm, ×8757 and ×46,760).* **Left:** *A subendothelial cell (*, at open arrow) lies abluminal to endothelium (e) and adluminal to an elastic lamina that is electron-dense (el). The processes of an interstitial fibroblast (if) extend around the vessel abluminal to the elastic lamina (arrow); transitional epithelial cell (ep).* **Right:** *At higher magnification the subendothelial cell (*) is identified as a pericyte, sharing the basal lamina of the endothelial cell (large double arrow), with few filaments in the midregion (illustrated) or in the extended cell processes. The peg and socket contact with the endothelial cell (small open arrow) is characteristic. Endothelial cells (e) and junction (open arrow), elastic lamina (el), interstitial fibroblast (if).*

organization, which clearly separates them from the capillary bed. In the fibroblast, tropoelastin induction and increased synthesis can be regulated by specific factors released locally by activated smooth muscle cells.[24] It is possible that the newly developing contractile cells express factors with a similar effect and that this leads to further tropelastin secretion by some fibroblasts that remain aligned around the vessel but are not incorporated into the wall. Around most vessels, however, fibroblasts are incorporated into the wall by forming an elastic lamina in the absence of contractile cells. In fetal bovine lung, Noguchi and colleagues[25] have reported the association between fibroblast expression of α-smooth muscle actin and tropoelastin secretion. Further studies are needed to establish the isoform present in migrating and aligning cells in the hyperoxic lung.

In part, normal wall structure determines the cell pathways responsible for wall reorganization in any given segment. Most fibroblasts are recruited to vessel segments where intermediate cells are not found, raising the question as to their location. Axial pathways—there are two per acinus—branch with several generations of alveolar ducts before dispersing into a capillary network in the alveolar wall. These show the typical transition from a fully to a partially muscular and then nonmuscular wall, and the nonmuscular region of these segments has a considerable intimal cell population that includes intermediate cells.[7] In the rat, as in the human lung, the axial segments have numerous side-branches (both conventional and supernumerary),[26] that quickly branch into a capillary network. These can arise directly from the muscular segment of the axial pathway but be nonmuscular at their origin. In the alveolar region of the normal lung, the many vessel profiles without intimal cells suggest either that not all axial pathways have the same cell arrangement or that these are the profiles of lateral branches. Although some overlap is likely the generalization seems justified that, in the hyperoxic lung, intermediate cells reorganize the walls of axial pathways and fibroblasts the numerous lateral ones. In fixative distended lungs, vessels < 30 μ in diameter can rarely be identified as arteries or veins, especially as both remodel in hyperoxia.[1,2,4] Thus, both small arteries and veins are included in the group of alveolar vessels analyzed here, and either could be reorganized through the cell pathways described. Neither cell pathway appears to reorganize the walls of larger alveolar vessels that by their structure are clearly identified as veins because in these vessels, fibroblasts are seen only external to the elastic lamina.

In summary, the walls of lung microvessels are reorganized by more than one cell pathway in hyperoxic pulmonary hypertension. The factors that drive the expression of a contractile cell phenotype are not known but could reflect the response to specific mediators, (eg,

platelet derived growth factor, granulocyte-macrophage colony stimulating factor, transforming growth factor β), changes in the interstitial collagen matrix, or physical forces transduced through the vessel wall.[21,27] Whether alveolar vessels ultimately develop uniform contractile cell populations that are all 'professional' smooth muscle cells in terms of the expression of their filament proteins or whether a range of cell types and subsets expressing a partial muscle phenotype, i.e., 'nonprofessional' contractile cells[28] persist in pulmonary hypertension will need to be established. Preliminary studies are underway to analyze this in the hyperoxic lung.

Acknowledgments

The author thanks Catherine Adler and Howard Mulhurn for expert technical assistance.

References

1. Jones R, Zapol WM, Reid L: Pulmonary artery remodelling and pulmonary hypertension after exposure to hyperoxia for 7 days: A morphometric and hemodynamic study. Am J Pathol 1984, 117:273–285
2. Jones R, Zapol WM, Reid L: Oxygen-toxicity and restructuring of pulmonary arteries: A morphometric study of the response to 4 weeks' exposure to hyperoxia and return to breathing air. Am J Pathol 1985, 121:212–223
3. Jones R, Zapol WM, Tomashefski JF, Kirton OC, Kobayashi K, Reid L: Pulmonary vascular pathology, Acute Respiratory Failure. Lung Biology in Health and Disease, Vol 24. Edited by WM Zapol, KJ Falke. New York, Marcel Dekker, 1985, pp 23–160
4. Hu LM, Jones R: Injury and remodeling of pulmonary veins by high oxygen: A morphometric study. Am J Pathol 1989, 134:253–262
5. Smith P, Heath D: Ultrastructure of hypoxic hypertensive pulmonary vascular disease. J Pathol 1977, 121:93–100
6. Meyrick BO, Reid L: Ultrastructural features of the distended pulmonary arteries of the normal rat. Anat Rec 1979, 193: 71–98
7. Davies P, Burke G, Reid L: The structure of the wall of the rat intraacinar pulmonary artery: An electron microscopic study of microdissected preparations. Microvasc Res 1986, 32: 50–63
8. Weibel ER. On pericytes, particularly their existence on lung capillaries. Microvasc Res 1974, 8:218–235
9. Sobin SS, Tremer HM, Hardy JD, Chiodi HP: Changes in arteriole in acute and chronic hypoxic pulmonary hypertension and recovery in rats. J Appl Physiol 1983, 55:1445–1455
10. Kistler GS, Caldwell PRB, Weibel ER: Development of fine damage to alveolar and capillary lining cells in oxygen-poisoned rat lungs. J Cell Biol 1967, 33:605–628
11. Bowden DH, Adamson IYR: Endothelial regeneration as a marker of the differential vascular response in oxygen-induced pulmonary edema. Lab Invest 1974, 30:350–357
12. Crapo JD, Peters-Golden M, Marsh-Salin J, Shelburne JS: Pathologic changes in the lungs of oxygen-adapted rats: A morphometric analysis. Lab Invest 1978, 39:640–653
13. Clark JG, Greenberg J: Modulation of the effects of alveolar macrophages on lung fibroblast collagen production rate. Am Rev Respir Dis 1987, 135:52–56
14. Peacock AJ, Dawes KE, Laurent GJ: Hypoxia stimulates endothelial cells (EC) to produce a growth factor and chemoattractant for lung fibroblasts (FB). Am Rev Respir Dis 1991, 143:A378
15. Tomsek JJ, Hay ED, Fujiwara K: Collagen modulates cell shape and cytoskeleton of embryonic corneal and fibroma fibroblasts: Distribution of actin, α-actinin and myosin. Dev Biol 1982, 92:107–122
16. Jones R, Adler C, Farber F: Lung vascular cell proliferation in hyperoxic pulmonary hypertension and on return to air: ^{3}H-thymidine pulse labeling of intimal, medial and adventitial cells in microvessels and at the hilum. Am Rev Respir Dis 1989, 140:1471–1477
17. Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR, Magno G: Granulation tissue as a contractile organ: A study of structure and function. J Exp Med 1972, 135:719–734
18. Berg JSV, Rudolph R, Poorlman WL, Disharoon DR: Comparative growth dynamics and actin concentration between cultured human myofibroblasts from granulating wounds and dermal fibroblasts from normal skin. Lab Invest 1989, 61:532–538
19. Jester JV, Rodrigues MM, Horman IM: Characterization of avascular corneal wound healing fibroblasts: New insights into the myofibroblast. Am J Pathol 1987, 127:140–148
20. Eddy RJ, Petro JA, Tomasek JJ: Evidence for the non-muscle nature of the "myofibroblast" of granulation tissue and hypotropic scar: An immunofluorescence study. Am J Pathol 1988, 130:252–260
21. Sappino AP, Schurch W, Gabbiani G: Differentiation repertoire of fibroblastic cells: Expression of cytoskeletal proteins as marker of phenotypic modulations. Lab Invest 1980, 63: 144–161
22. Meyrick BO, Reid L: The effect of continued hypoxia on rat pulmonary arterial circulation: An ultrastructural study. Lab Invest 1978, 38:188–200
23. Sims DE: The pericyte: A review. Tissue Cell 1986, 18:153–174
24. Mecham RP, Whitehouse LA, Wrenn DS, Parks WC, Griffen GL, Senior RM, Crouch EC, Stenmark KR, Voelkel NF: Smooth muscle-mediated connective tissue remodeling in pulmonary hypertension. Science 1987, 237:423–426
25. Noguchi A, Reddy R, Kursar JD, Parks WC, Mecham RP: Smooth muscle isoactin and elastin in fetal bovine lung. Exp Lung Res 1989, 15:537–552
26. Hislop A, Reid LR: Normal structure and dimensions of the pulmonary arteries in the rat. J Anat 1978, 125:71–83
27. Ingber DE, Jamieson JD: Cells as tensegrity structures: Architectural regulation of histodifferentiation by physical forces transduced over the basement membrane, Gene Expression During Normal and Malignant Differentiation. Edited by Aderson LC, Gahmberg CG, Ekblom P. London, Academic Press, 1985, p 13
28. Schmidt RA, Gown A: Perspective "professional" and "nonprofessional" contractile cells in the lung. Am J Respir Mol Biol 1980, 3:513–514