Hypoxia and Incorporation of ^{3}H-Thymidine by Cells of the Rat Pulmonary Arteries and Alveolar Wall

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In the pulmonary arterial circulation hypoxia produces increase in thickness of the medial muscle coat as well as of the adventitia; in addition, muscle appears in smaller arteries than is normal and the number of small arteries that fill on Micropaque-gelatin injection is reduced. To assess the role of hyperplasia in these changes, the uptake of ^{3}H-thymidine by the cells of the pulmonary arterial wall has been studied in rats exposed to hypobaric hypoxia (exposure to 380 torr) after 1, 3, 5, 7, 10, and 14 days. Using autoradiographs of 1-μm sections, the glutaraldehyde-distended intrapulmonary hilar muscular artery, the peripheral, intraacinar arteries less than 100 μm in external diameter, and the alveolar wall had different patterns of uptake. In the hilar pulmonary artery, after 24 hours of exposure, the labeling index for adventitial fibroblasts is increased eightfold over the control value, and for endothelial cells, threefold, while for medial smooth muscle cells, there is a gradual and small increase to Day 14. Newly muscularized intraacinar arteries are first apparent at Day 3, when they comprise 40% of the intraacinar arteries, increasing to 80% at Day 7. No decrease in density of arteries is found. Uptake of ^{3}H-thymidine by new muscle cells is not apparent until Day 5 when labeling is maximum. The endothelial cells of the newly muscularized arteries show an increased labeling index only at Days 7 and 10. The veins and normally muscular arteries do not show these changes. In the alveolar walls, the concentration of labeled cells is significantly above the control value at Days 3, 5, and 7 and significantly below, at Day 14. At this level, the interstitial, epithelial, and endothelial cells contribute to the increase. (Am J Pathol 96:51–70, 1979)

INCREASED MEDIAL THICKNESS of the pulmonary arteries is a well established feature of chronic hypoxia both in humans [1–4] and in rats.[5–8] More recently, reduction in number of small arteries that are filled in Micropaque-gelatin injected lungs [3,4,7–10] and extension of muscle into smaller and more peripheral arteries than is normal have been described.[6–8,10–12] Ultrastructural studies have shown that, in the hypoxic rat, extension is found as early as the second day of exposure and that the new muscle cells develop by differentiation of precursor smooth muscle cells, normally present in the nonmuscular regions of artery,[13–17] the pericyte of the nonmuscular arteries, and the intermediate cell of the partially muscular arteries.[15,18] A striking increase in the thickness of the dense adventitial layer of the rat hilar muscular pulmonary artery has also been reported.[14]

After exposure to hypoxia, Volkel and colleagues [19] have reported a

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350% increase in the incorporation of ^{3}H-thymidine into total rat lung tissue DNA, indicating hyperplasia of lung cells, although those responsible were not fully identified. The present study is concerned with the contribution of hyperplasia to the hypoxia-induced pulmonary arterial changes. In autoradiographs of 1-μm sections, we have traced the uptake of ^{3}H-thymidine, and so of mitosis, by the cells of the wall of the pulmonary arterial tree a) in the hilar muscular artery and b) in the peripheral intraacinar arteries of less than 100 μm external diameter, which in the hypoxic animals includes both newly muscularized and normally muscular arteries; uptake by the cells of the alveolar wall also has been examined.

Materials and Methods

Thirty-six male Sprague–Dawley rats (Charles River, Portage, Mich), 18 control and 18 experimental, and weighing approximately 230 g at the start of the experiment, were used in this study. The experimental or "hypoxic" animals were exposed to low barometric pressure in a Wright's hypobaric chamber.[20] To allow the animals to acclimatize to low pressure, for the first 24 hours of exposure, the pressure within the chamber was reduced only to 500 torr and from the second day onwards, to 380 torr, equivalent to approximately 5500 m above sea level. For 15 minutes each day, the pressure within the chamber was returned to normal so that food and water could be replenished and the cages cleaned.

Three control and three experimental animals were killed after 24 hours of exposure and after 3, 5, 7, 10, and 14 days; these times will be referred to as Days 1, 3, 5, 7, 10, and 14, respectively. All the animals were weighed at the start of the experiment and on each day that animals were killed.

On the day of killing, each animal to be killed, whether in the control or hypoxic group, was injected intraperitoneally with 2 μCi/g of body weight of thymidine (methyl-^{3}H), specific activity 40–60 Ci/mmol (New England Nuclear Co). The first animal was injected at 10:00 AM and the remaining five at 15-minute intervals. One hour after injection the animals were heavily anesthetized by an intraperitoneal injection of pentobarbitone sodium (60 mg/200 g of body weight). While the animals were still breathing, the lungs, heart, and trachea were removed intact through a median sternotomy. To ensure distension of the pulmonary circulation and of the alveoli, after tying the pulmonary veins at the hilum, the lungs were fixed by simultaneously injecting 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) into the pulmonary trunk and trachea at a pressure of 100 cm and 25 cm of water, respectively.[13,14,21] The tissue was then left for 2 hours before dissection.

Since our previous studies [7,8,14] have shown that hypoxia produces the same structural changes in all lobes of the lung, only the left lung was examined in the present study. Blocks, 2–3 cu mm, were taken from two levels of pulmonary artery, the hilum and the periphery. Two blocks of the hilar artery were taken; these included the first 4 mm of the intrapulmonary pathway, each block being 2 mm thick. This level represents the population of large muscular arteries. The blocks of lung periphery were taken approximately 2 mm in from the diaphragmatic surface and included mostly intraacinar arteries less than 100 μm in external diameter which are of a "mixed population," muscular, partially muscular, and nonmuscular.

The blocks were fixed in glutaraldehyde for a further 90 minutes, washed in cacodylate buffer overnight at 4 C, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon/Araldite mixture. Sections, 1 μm thick, were cut using an LKB Ultrotome III and

glass knives. Three or four sections from each block were mounted onto acid-cleaned glass slides. The slides were dipped in Kodak NTB_2 emulsion diluted 1 : 1 with distilled water at 43 C and exposed for 2 weeks at 4 C. The autoradiographs were developed in Kodak Dektol diluted 1 : 1, fixed in Kodak Ektoflo diluted 1 : 8, both at 15 C, and stained with 0.5% toluidine blue at 60 C for 1 minute. Control autoradiographs of unlabeled tissue showed background counts to be negligible.

Quantitation

In one section from each of two blocks from each animal, medial and adventitial thickness of the hilar pulmonary artery was measured in at least three regions of wall, the sections of tissue cut obliquely being avoided. Medial thickness was measured from, and included, the internal and external elastic laminas. Adventitial thickness was measured from the outside of the external elastic lamina to the outer margin of the dense collagenous layer. Mean medial and adventitial thickness was calculated for each animal and for each group of animals, the standard error of the mean (SE) was calculated, and the Student *t* test applied between groups. The length of wall included in each section was also measured, and the area of media and adventitia included in each section was estimated.

In each section, using an oil immersion lens, all the nuclei (that is of endothelial cells, smooth muscle cells, and adventitial fibroblasts) of the entire wall of the hilar pulmonary artery were counted. This gave a total of at least 1000 cells per animal; for control animals this included not less than 155 endothelial cells, 582 smooth muscle cells, and 254 adventitial fibroblasts. In addition, the total number of labeled nuclei (that is a nucleus with 5 or more silver grains) for each cell population was noted.

For examination of the peripheral intraacinar arteries, an area of at least 6 sq mm of peripheral lung was examined from each animal and included one section from at least two blocks. In these sections, the structure and external diameter of each vessel of less than 100 μm was noted. The total number of endothelial cell nuclei and the number labeled was recorded for each vessel, as was the total number of new smooth muscle cells and, where applicable, those labeled. New smooth muscle cells were distinguished from normal smooth muscle cells because they were internal to a single elastic lamina; normal arterial smooth muscle lies between an internal and external elastic lamina. For each cell type, at both levels of artery examined, ie, the hilar and intraacinar arteries, the total number of cells counted and the proportion labeled, in each group of animals at each exposure time, was pooled and the standard deviation (SD) was calculated.

On a separate occasion, the same slides of peripheral lung tissue were examined again and all labeled cells, including those of the blood vessels, were analyzed. The labeled cells were divided into six categories: 1) interstitial cells including fibroblasts, pericytes, contractile interstitial cells, and interstitial monocytes, 2) capillary endothelium, 3) alveolar epithelium including Types I, II, and III pneumonocytes, 4) cells in the walls of blood vessels greater than 15 μm external diameter, both arteries and veins, including endothelial cells, normal and new smooth muscle cells, and adventitial fibroblasts, 5) intracapillary monocytes, and 6) all other labeled cells not identified as belonging to the other categories, including alveolar space macrophages. Virtually all cells in 6) fell in the latter group. The number of labeled cells in each category for each group of animals at each exposure time was pooled and related to the area of tissue examined and the SD for each group, calculated.

Assessment of Right Ventricular Hypertrophy

After fixation in glutaraldehyde for at least 7 days, the heart was dissected and weighed by the method of Fulton, Hutchinson, and Jones,[22] and the ratio of left ventricle plus septum to right ventricle was calculated.

Results

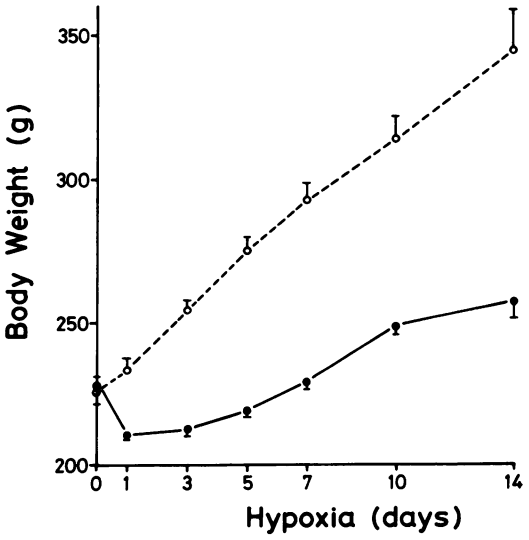
Body Weight and Assessment of Right Ventricular Hypertrophy

After only one day's exposure to hypoxia, the animals lose weight (Text-figure 1) but by Day 7 have regained their original weight; at Day 14, the animals weigh approximately 80 g less than the controls. At all times studied, except for Day 1, total heart weight of the hypoxic animals is less than that of the controls but not significantly except at Day 3 (Table 1). Absolute weight of the right ventricle is significantly increased from Day 7 ($P = <0.05$) and by Day 5, right ventricular hypertrophy (LV+S/RV) is significant in the hypoxic animals ($P = <0.05$) (as in References 7, 8, 14).

Hilar Pulmonary Artery

Medial and Adventitial Thickness

Hypoxia causes an absolute increase in both medial and adventitial thickness in the hilar pulmonary artery. In control animals medial thickness has a mean value of 11.83 μm (SE $\pm$ 0.70). After one day's hypoxia, medial thickness is within the normal range, is significantly increased after Day 3 ($P = < 0.001$), continues to increase to Day 10, and is similar at Day 14, when the thickness is more than double the control value (Text-figure 2). Adventitial thickness also is normal at Day 1, significantly increased at Day 3 ($P = < 0.001$), continues to increase to Day 7, when it shows a threefold increase, and from this time remains steady (Text-figure 2).

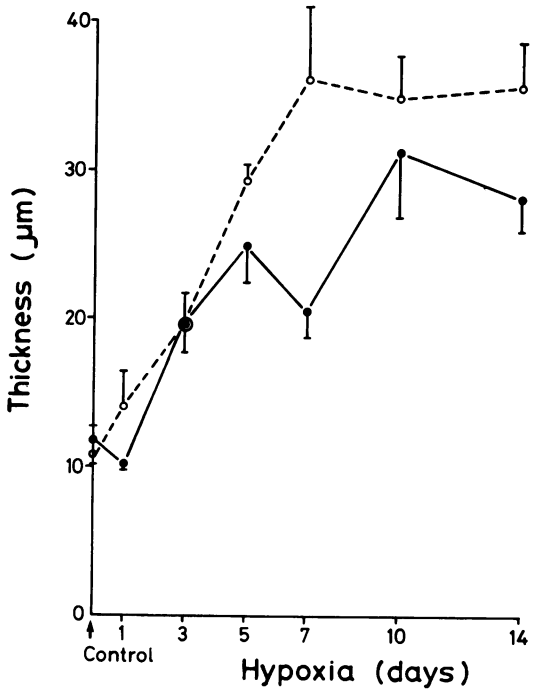


TEXT-FIGURE 1—Mean body weight of control (*open circles*) and hypoxic (*solid circles*) animals for periods to 14 days.

Table 1—Total Heart and Right Ventricular Weights, and Ratio of Left Ventricle + Septum (LV + S) to Right Ventricle (RV) After Exposure to Hypoxia

Days of hypoxia	No. of animals	Total heart weight (g)				Right ventricle (g)				LV +S/RV			
		Control		Hypoxic		Control		Hypoxic		Control		Hypoxic	
		m	SE	m	SE	m	SE	m	SE	m	SE	m	SE
1	3	0.71	0.03	0.71	0.05	0.15	0.01	0.15	0.01	3.88	0.19	3.72	0.19
3	3	0.76	0.01	0.65	0.02*	0.18	0.06	0.17	0.02	3.32	0.19	2.92	0.32
5	3	0.73	0.05	0.71	0.04	0.17	0.01	0.19	0.01	3.33	0.13	2.71	0.14*
7	3	0.77	0.03	0.75	0.03	0.19	0.01	0.21	0.01*	3.17	0.11	2.50	0.11†
10	3	0.85	0.04	0.76	0.03	0.19	0.02	0.24	0.01*	3.44	0.19	2.23	0.19*
14	3	0.96	0.04	0.85	0.06	0.22	0.01	0.28	0.02*	3.43	0.31	2.03	0.09†

* $P = < 0.05$ – Student t test
† $P = < 0.01$

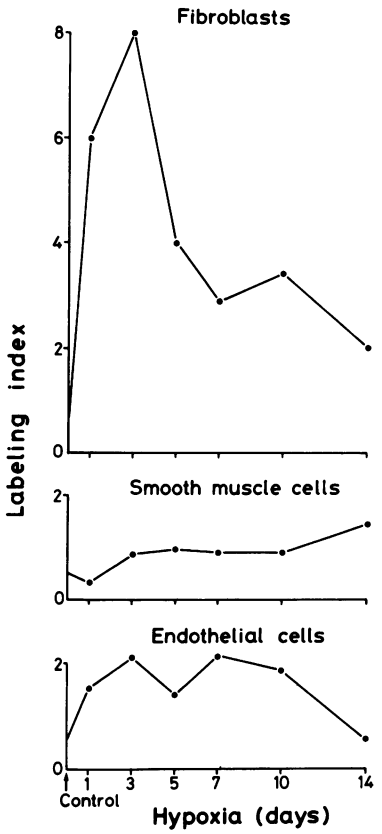


TEXT-FIGURE 2—Medial (*solid circles*) and adventitial (*open circles*) thickness of the hilar muscular artery after exposure to hypoxia (SE is given).

Uptake of ^{3}H-Thymidine

In control animals in the hilar pulmonary artery the labeling index (percentage of the population of a given cell type that is labeled by ^{3}H-thymidine) for endothelial cells is 0.54 (turnover time about 7.5 days), for medial smooth muscle cells, 0.50 (turnover time about 7.5 days), and for adventitial fibroblasts, 0.69 (turnover time about 4.5 days). Although it was thought that, with hypoxia, the smooth muscle cell would be the cell to show most active incorporation of ^{3}H-thymidine, at the hilum, it is the adventitial fibroblasts and endothelial cells that show the greatest and earliest activity.

After one day's exposure to hypoxia, the adventitial fibroblasts show an eightfold increase in their labeling index and at Day 3, an 11-fold; thereafter, the index falls to Day 14 when a threefold increase is still apparent (Figure 1A and Text-figure 3). Between Days 1 and 10 of hypoxia, the labeling index for the endothelial cells is increased two- or threefold but by Day 14 is again within the normal range (Figure 1A and Text-figure 3). For the smooth muscle cells, although the labeling index increases steadily it is only at Day 14 that the index is doubled (Figure 1B and Text-figure 3). At Days 7 and 10, the majority of labeled smooth muscle cells



TEXT-FIGURE 3—Labeling index of adventitial fibroblasts, medial smooth muscle cells, and endothelial cells of the hilar muscular artery after exposure to hypoxia (SD too small to be shown).

were in the inner half of the wall, while at Day 14, increased uptake occurred throughout the wall.

Increase in Nuclear Number

After exposure to hypoxia, from light microscopic inspection of slides, hyperplasia of fibroblasts and endothelial cells is obvious, while the number of smooth muscle cells appears little changed. An estimate of the relative increase in cell number was obtained, using the ratios of either total number of fibroblast or endothelial cell nuclei per animal to total number of smooth muscle cell nuclei per animal (Table 2). The mean nuclear ratio of fibroblasts to smooth muscle cells in the control animals is 0.46 (SE ± 0.02): From Day 3, there is a significant increase in the ratio, and from Day 5 onwards, a doubling. The mean ratio of endothelial cells to smooth muscle cells in the control animals is less, 0.28 (SE ± 0.01): At Days 7, 10, and 14, the ratio is significantly increased.

Table 2—Ratio of Nuclei of Adventitial Fibroblasts and of Endothelial Cells to Smooth Muscle Cells in the Hilar Pulmonary Artery after Exposure to Hypoxia

Days of hypoxia	No. of animals	Fibroblasts: smooth muscle		Endothelial cell: smooth muscle	
		m	SE	m	SE
Control	18	0.46	0.02	0.28	0.01
1	3	0.56	0.05	0.31	0.02
3	3	0.62	0.02*	0.27	0.01
5	3	1.04	0.08*	0.31	0.36
7	3	1.03	0.12*	0.44	0.04*
10	3	1.01	0.15†	0.47	0.06†
14	3	0.75	0.05*	0.33	0.01*

* $P = < 0.01$—Student t test
† $P = < 0.001$

Concentration of Cells

An estimate of cellular concentration was obtained by relating the number of smooth muscle cells and fibroblasts to unit area of media and adventitia, respectively. The mean number of smooth muscle cells per unit of media in control animals is 83.32 (SE ± 4.31). From Day 3 of hypoxia, the number is significantly decreased ($P = < 0.05$) and decreases further to Day 7 ($P = < 0.001$) when the number is halved; Days 7, 10, and 14 are similar (Table 3). The number of adventitial fibroblasts per unit area in control animals is 44.31 (SE ± 6.29). This number also decreases with hypoxia, although the decrease is not statistically significant until Day 7 ($P = < 0.01$). That the concentration of both smooth muscle cells and fibroblasts decreases suggests that hypertrophy of the cell

Table 3—Number of Smooth Muscle Cells and Fibroblasts Per Unit Area (sq mm) of Media and Adventitia

Days of hypoxia	No. of animals	Smooth muscle		Fibroblasts	
		m	SE	m	SE
Control	18	83.32	4.31	44.31	6.29
1	3	87.11	5.42	37.42	8.07
3	3	57.11	8.80*	35.77	1.17
5	3	49.31	4.88‡	43.09	4.17
7	3	39.84	7.06‡	22.80	3.51†
10	3	36.57	3.26‡	32.47	5.05
14	3	39.93	3.84‡	23.85	3.30†

* $P = < 0.05$—Student t test
† $P = < 0.01$
‡ $P = < 0.001$

and/or increase in intercellular connective tissue, as well as hyperplasia, contribute to the thickening.

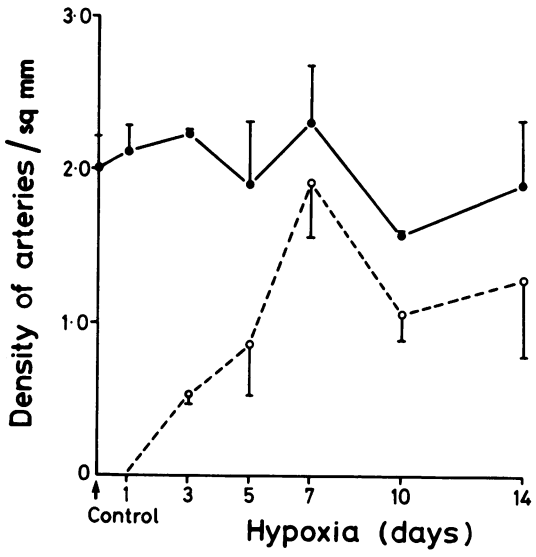
Peripheral Intraacinar Arteries

Density of Arteries

In a sq mm of peripheral lung tissue—this is roughly the area of one acinus—approximately two arteries between 15 and 100 μm external diameter are found. With exposure to hypoxia, the density of arteries remains similar to that of the controls (Text-figure 4), the trend to a decrease in number seen at Days 10 and 14 is probably more a reflection of increased alveolar diameter. Within the normal population of peripheral arteries, newly muscularized arteries appear. They are first evident at Day 3 when they comprise about 40% of the total arteries but, after Day 7, they constitute 80% of arteries (Text-figure 4); at Days 10 and 14, the density of newly muscularized arteries is lower (1.1–1.3/sq mm; 63%). This decrease in density is probably due to the maturation of some of the newly muscularized arteries that, by laying down an internal elastic lamina, become indistinguishable from a normally muscular artery.

Uptake of ^{3}H-Thymidine

From the third day of exposure onwards all the animals studied were affected by the new muscularization. Although new muscle cells are first apparent at Day 3, incorporation of ^{3}H-thymidine by new muscle cells is



TEXT-FIGURE 4—Density of total intraacinar arteries (*solid circles*) and of newly muscularized arteries (*open circles*) per square millimeter after exposure to hypoxia (SE is given).

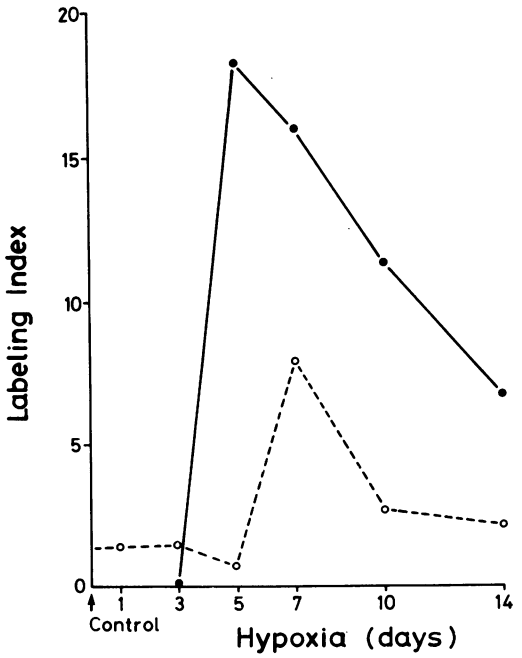
not evident until Day 5 (Text-figure 5 and Figures 2A and B), when 18% of the total new muscle cells are labeled; at Days 7, 10, and 14, there is a progressive fall in the labeling index of these cells.

The labeling index of the endothelial cells associated with peripheral arteries in the control animals is 1.41. With hypoxia, no increase in the labeling index of endothelial cells is apparent until Day 7, when it is doubled, but by Day 14 it has fallen to normal levels (Text-figure 5). No increase in labeling index was detected for the peripheral venous endothelial cells.

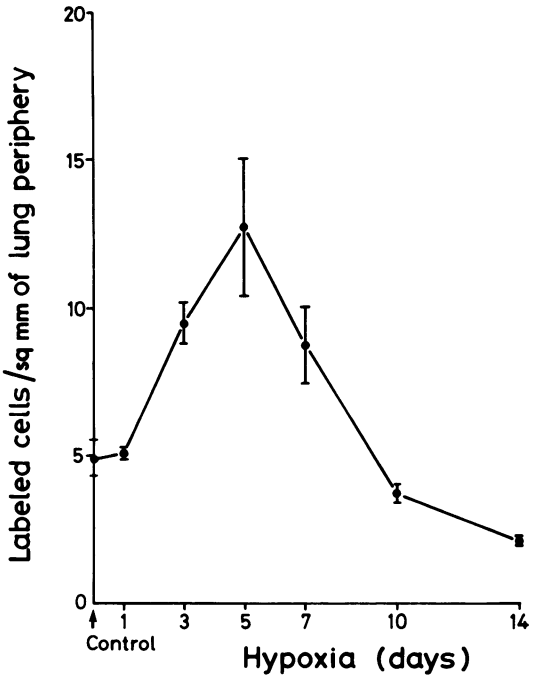
In the few normally muscular arteries encountered at the periphery (14 in control and 27 in hypoxic animals), there was no increase in the frequency of labeled smooth muscle cells. However, twice as many adventitial fibroblasts were labeled in the hypoxic animals as in the controls. It seems, therefore, that the same pattern of uptake of ^{3}H-thymidine as described in the hilar muscular pulmonary artery occurs in all muscular arteries.

Alveolar Wall

In control animals, there are approximately 5 labeled alveolar wall cells per sq mm (m = 4.89; SE ± 0.59; Text-figure 6). After one day of hypoxia,



TEXT-FIGURE 5—Labeling index of intraacinar new muscle cells (*solid circles*) and arterial endothelial cells (*open circles*) after exposure to hypoxia (SD too small to be shown).



TEXT-FIGURE 6—Total number of labeled cells per square millimeter of lung periphery, that is alveolar walls, after exposure to hypoxia (SE is given).

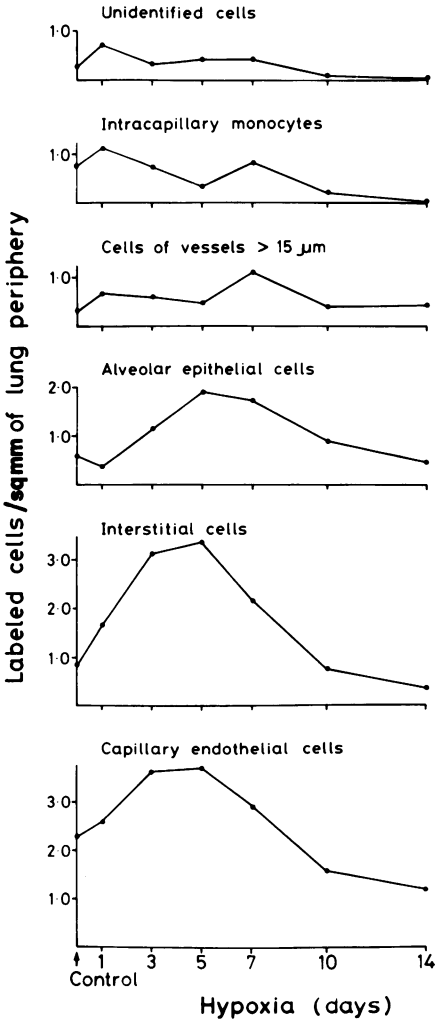
no increase in labeled cells is seen; by Day 3, there is a significant increase ($P = < 0.001$) with a further increase to Day 5 when the maximum of more than a twofold increase is found. From Day 5, labeling decreases so that at Day 10 it is within the normal range and at Day 14, is significantly below normal ($P = < 0.001$).

For the six cell categories the increase in number of labeled cells is first apparent in the interstitial and capillary endothelial categories and later in the alveolar epithelial. At Day 1, the distribution of labeled cells in the six categories is similar to the controls (Text-figure 7). At Day 3, the interstitial cells show approximately a fourfold increase above the control value (0.80) that continues somewhat to Day 5 and then falls, reaching normal or values just below normal from Day 10. The timing and pattern of increase is similar for the capillary endothelial cells, although for this cell type the maximum increase above control value (2.27) is approximately 66% and from Day 10, the number is below normal. In control animals the number of labeled alveolar epithelial cells (0.58) is lower than the other two cell types, and the increase of approximately threefold is at a slightly later time, at Days 5 and 7; from Day 7, there is a fall in the number of labeled epithelial cells, which returns to normal levels by Day 10. The number of labeled cells in the walls of blood vessels greater than 15

μm in diameter increases above control value (0.32) only at Days 3 and 7. The number of labeled intracapillary monocytes decreases progressively from control value (0.77) to zero at Day 14. The number of labeled cells in the group of "unidentified" cells is similar to the control value (0.27) throughout the experiment (Text-figure 7).

Discussion

Our previous studies with pulmonary hypertension induced by exposure to hypobaric hypoxia [7,8,14,23] have demonstrated the nature of the response at the various levels of the arterial tree. In the muscular arteries,



TEXT-FIGURE 7—Number of labeled capillary endothelial cells, interstitial cells, alveolar epithelial cells, cells in vessel walls greater than 15 μm diameter, intracapillary monocytes, and unidentified cells per square millimeter of lung periphery after exposure to hypoxia (SD too small to be shown).

the medial and adventitial coats thicken while in the smaller, more peripheral arteries, new muscle appears in normally nonmuscular regions, with a reduction in lumen diameter. The present study, designed to elucidate the role of hyperplasia in these changes, has shown differences in timing and severity of mitotic activity for the various cell types and, in some instances, for a given cell type at various levels.

This present study has also shown that in the hilar pulmonary artery, the normal turnover time of the endothelial and smooth muscle cells is similar, about 7.5 days, while that of the adventitial fibroblasts is faster, about 4.5 days. Despite the increase in medial thickness of the muscular pulmonary arteries, apparent from Day 3 of hypoxia and reaching maximum at Day 10, there is only a small and late increase in mitotic activity—even at Day 14 the increase is less than threefold. It is unlikely that with longer exposure times a more marked hyperplasia would occur since our light [7] and electron microscopic studies [14] of long-term exposure (8 weeks) show no further increase in medial thickness. During the first days of hypoxia our study shows a reduction in concentration of smooth muscle cells per unit, which is additional evidence that at this time the increase in medial thickness is the result of smooth muscle hypertrophy. Later, at Day 28, increased amounts of intercellular connective tissue also contribute.[14,24]

In previous reports some authors have suggested that the increase in medial thickness was the result of smooth muscle hypertrophy [12] while others favored hyperplasia.[25] In human primary and thromboembolic pulmonary hypertension, any increase in medial thickness of the muscular pulmonary arteries was considered to be due to hypertrophy of smooth muscle cells rather than hyperplasia, since nuclear number per unit length of wall was normal although medial thickness was increased.[9]

One of the most striking findings in our study is in the hilar muscular artery which, from the first day of exposure, shows a dramatic eightfold increase in mitotic activity of the adventitial fibroblasts and a smaller, threefold increase in that of the endothelial cells. In our earlier experiments we reported hypertrophy of adventitial fibroblasts [13] and endothelial cells [14]; although not apparent at Day 1, by Day 3 increased cell diameter, together with increased amounts of rough endoplasmic reticulum and an extensive Golgi apparatus, were apparent. When mitosis is stimulated above basal levels, hypertrophy of the cell usually precedes [26] and may itself be the trigger for mitosis. It may be that either the necessary degree of hypertrophy is achieved more rapidly in the fibroblasts and endothelial cell, or that a lesser degree of hypertrophy suffices for the fibroblasts and endothelial cell than for the smooth muscle cell.

From inspection of microscopic tissue, hyperplasia of adventitial fibroblasts has been described in bovine lungs at an altitude of 4527 m,[27] in the lungs of dogs with systemic to pulmonary artery anastomosis [28] and in several cases of human hypoxic disease.[29,30] More recently, adventitial proliferation has been reported in rats with Crotalaria-induced pulmonary hypertension.[31]

The other striking change is at the periphery—the appearance of newly muscularized arteries. Newly muscularized arteries, that is arteries with identifiable muscle precursor cells internal to a single elastic lamina, were frequently identified from Day 3 of exposure, but uptake of ^{3}H-thymidine was not detected until Day 5. The appearance of new muscle in normally nonmuscular regions of peripheral intraacinar arteries, that is, in the size range of the "mixed population" of arteries, was first thought to arise from multiplication of smooth muscle cells in the peripheral part of a pathway where smooth muscle cells are arranged as a spiral.[7] By electron microscopy, we have demonstrated that the new muscle represents hypertrophy and metaplasia of precursor smooth muscle cells normally present in the nonmuscular regions of artery but not apparent by light microscopy.[13,14,16,17] In the nonmuscular artery the precursor cell is the pericyte and in the partially muscular artery, the intermediate cell.[13,15] By ultrastructural examination precursor cells were seen in mitosis at Days 7 and 10.[14] Taking together our earlier ultrastructural observations and the present study of mitosis, it seems that when the precursor cell enters mitosis it has the characteristics of an intermediate cell (ie, one intermediate in structure between a pericyte and smooth muscle cell). The pericyte differentiates to an intermediate cell before division, and it seems that only after an intermediate cell has divided does it mature to a smooth muscle cell. In skeletal muscle, it is only the myoblasts, the penultimate generation of cell, that divide to form myotubes.[32]

The mitotic activity of the endothelial cells is different at the various levels of artery studied. The onset of mitotic activity of the endothelial cell in the newly muscularized arteries with exposure to hypoxia is later than for the endothelial cell at the hilum, although its degree is similar and is later than in the alveolar capillaries, but the degree is more marked. It has been shown that endothelial cell structure differs at the various levels of artery studied; for example, in the normal rat, the number of Weibel–Palade bodies decreases as the artery passes distally, and in the alveolar capillaries, are rare.[14,33] Thus, the endothelial cells at each level probably have different intrinsic properties and may respond differently to a given stimulus.

With hypoxia, a reduction in concentration of arteries that are filled

with Micropaque has been reported.[7,8] Throughout the present study the density of arteries varied little. With exposure to hypoxia for periods of up to 14 days, it seems that there is not a real loss of arteries; rather, because of reduction in lumen diameter, penetration of the injection medium stops at an arterial level more proximal in position than in normal lung. Encroachment of new muscle on the lumen and endothelial swelling have been suggested to contribute to the loss of filling,[14] and the present study indicates, as additional factors, proliferation of the endothelial cell and hypertrophy and proliferation of the intermediate cell and pericyte.

It may be that, for capillaries, the alveolar interstitial cells are the counterpart of the adventitial fibroblast of the muscular arteries. The increase in mitotic activity of "alveolar fibroblasts" is, however, at a later time and is less marked than the fibroblasts of the muscular arteries.

The increase in mitotic activity of the alveolar epithelial cells with exposure to hypoxia is due mainly to uptake of ^{3}H-thymidine by the Type II pneumonocytes although occasional Type I pneumonocytes were labeled. This confirms earlier reports that demonstrate proliferation of Type II pneumonocytes and their eventual thinning to Type I pneumonocytes when epithelial damage occurs, for example, after inhalation of ozone,[34] nitrogen dioxide,[35] and 100% oxygen.[36] Electron-dense Type I pneumonocytes have been noted from Day 5 of hypoxia.[13]

In a previous study we have reported that the percentage of Type III pneumonocytes contributing to the alveolar lining is reduced with 24 hours of exposure to hypoxia.[37] This reduction in number was apparently due to disappearance of the brush border and extension of a Type I pneumonocyte cytoplasmic process along the alveolar surface of the Type III. Since in the present study no uptake of ^{3}H-thymidine was seen for the Type III pneumonocyte, this explanation still seems probable. Type III pneumonocytes are still reduced in number at Day 3 of hypoxia but by Day 5 are restored to their normal appearance and are again present in normal numbers.[13] The nature of the labeled intracapillary monocytes and why they are no longer apparent at Day 14 is obscure.

That lung volume increases with exposure to hypoxia is well established.[7,38,39] The increased size is thought to be the result of enlargement of alveoli and alveolar ducts rather than any increase in number. *In vitro* techniques have shown that incorporation of ^{3}H-thymidine by slices of lung tissue from rats exposed to hypoxia (440 torr) for various times is increased by approximately 350% after the ninth day of exposure.[19] From a preliminary qualitative study, these authors suggest that the increase is due to greater labeling of cells in the "mesenchyme" of the walls of the small pulmonary arteries and of bronchial tissue. The present study shows

a similar maximal increase in uptake, 250%, by the cells at alveolar level, but at an earlier time (Day 5); this is perhaps the result of the more severe degree of hypoxia used in our experiments. Although increased uptake by the fibroblasts in the walls of the muscular pulmonary arteries and their accompanying airways was found in the present study, our quantitative analysis of the cells that are responsible shows that the increased activity is due mainly to the interstitial and endothelial cells of the alveolar wall, and to the alveolar epithelial cells.

The role of hypoxia, *per se*, and of the rise in pulmonary artery pressure it produces are probably different for the various levels and various cell types of the pulmonary arterial tree. The reason for proliferation may be to replace a damaged cell, a response to work hypertrophy, or the effect of a mitogenic agent. It is of interest that insulin, lipoproteins, platelet factor, and thrombin have all been suggested to have a mitogenic effect. With hypoxia, platelets have been demonstrated in the walls of the hilar and intraacinar arteries.[13,14] Alternatively, receptors to mediators, such as norepinephrine, acetylcholine, angiotensin II, histamine, and serotonin, present on the cell surface, when activated, may initiate multiplication.

The immediate response to hypoxia is peripheral vasoconstriction of muscular arteries causing an increase in pulmonary artery pressure and pulmonary vascular resistance. The changes in the hilar artery are probably the result of this hemodynamic change, the early and dramatic increase in mitotic activity of the adventitial fibroblasts being a response to increase in transmural pressure. In the unanesthetized rat, a small but significant increase in pulmonary artery pressure has been found after just 10 minutes' exposure (Rabinovitch M, personal communication). The similar timing of the increase in mitotic activity of the endothelial cell without evidence of its injury suggests that its multiplication also can reasonably be related to the pressure increase.

Mitosis of the arterial smooth muscle cells seems a response to the duration and level of hypertension. In the present study, mitotic activity continues to increase slowly and steadily to Day 14, when the value is slightly more than double. Pulmonary artery pressure of hypoxic rats, measured using an indwelling catheter, behaves in a similar way.[23] In the rabbit with systemic hypertension produced by applying a ligature to the abdominal aorta, Bevan [41] has shown that the time course of systemic pressure increase is similar to the rate of smooth muscle proliferation in the common carotid artery, aorta, and muscular splenic arteries. Additionally, in rats fed with Crotalaria spectabilis seeds, medial and adventitial thickening are found without hypoxemia [31]; here, too, thickening follows increase in pressure.[42] In the intraacinar arteries and the alveolar wall,

hypoxia is likely to be more important than at the hilum since, even under conditions of maximum desaturation of mixed venous blood, the partially muscular and nonmuscular arteries as well as the alveolar capillaries will be 100% saturated with oxygen. These vessels are beyond the small muscular arteries in which there is relatively most muscle in the circulation and in which hypoxic vasoconstriction occurs. Constriction of the more proximal muscular arteries will alter the pattern of flow through the small distal vessel where change in flow rate and degree of distension could contribute. Although the spiral of muscle in the partially muscular region is able to constrict,[43] whether or not it does in hypoxia has not been established. In either case, the endothelial cell of the partially muscular artery behaves like that of the nonmuscular artery and alveolar capillary. In all of these structures the endothelial cell is edematous [14] suggesting that, at least as regards endothelium, these structures are similar. In our previous study we had suggested that the pulmonary artery tree has three compartments divided according to size: Compartment 1, the large muscular pulmonary arteries; Compartment 2, the "mixed population" of arteries less than 100 μm in diameter; and Compartment 3, the alveolar capillaries, less than 15 μm in diameter.[14] From the present study it seems that the compartments in the pulmonary artery might be modified slightly to accommodate structure as well as diameter—Compartment 1 being all the muscular arteries; Compartment 2, the partially and nonmuscular arteries less than 100 μm in diameter; and Compartment 3, the alveolar "capillaries" less than 15 μm. In addition, the present study suggests a fourth compartment, the intraacinar veins, since the endothelial cells in this compartment show no increase in mitotic activity.

The endothelial damage could be directly from hypoxia and, for the changes at the periphery, it seems justified to suggest that this cell has an important role. Whether directly or through its effect on the endothelial cell, hypoxia quickly produces hypertrophy of the intermediate cell and pericyte. Perhaps a mediator released from the endothelial cell causes hypertrophy of precursor smooth muscle cells. The stimulus to mitosis in these precursor cells could then be hypertrophy. The alveolar epithelial damage could be either from low pressure or hypoxia, and here the reason for proliferation seems to be the need for cell replacement.

The way that hypoxic vasoconstriction is brought about is still unclear. Cells in the walls of arteries at intraacinar level are well placed to trigger this response either by production of a humoral mediator or, if cell membrane state changes, by direct propagation of this to the smooth muscle of the small muscular arteries. Whatever the reason, hypoxia starts a chain of events by which large arteries and the microcirculation of the

lung are remodeled, by metaplasia and increased mitotic activity of cells including endothelial cells, precursor smooth muscle cells, and fibroblasts of adventitia and of alveolar walls; paradoxically, least proliferation is found in the smooth muscle cells of the medial coat.

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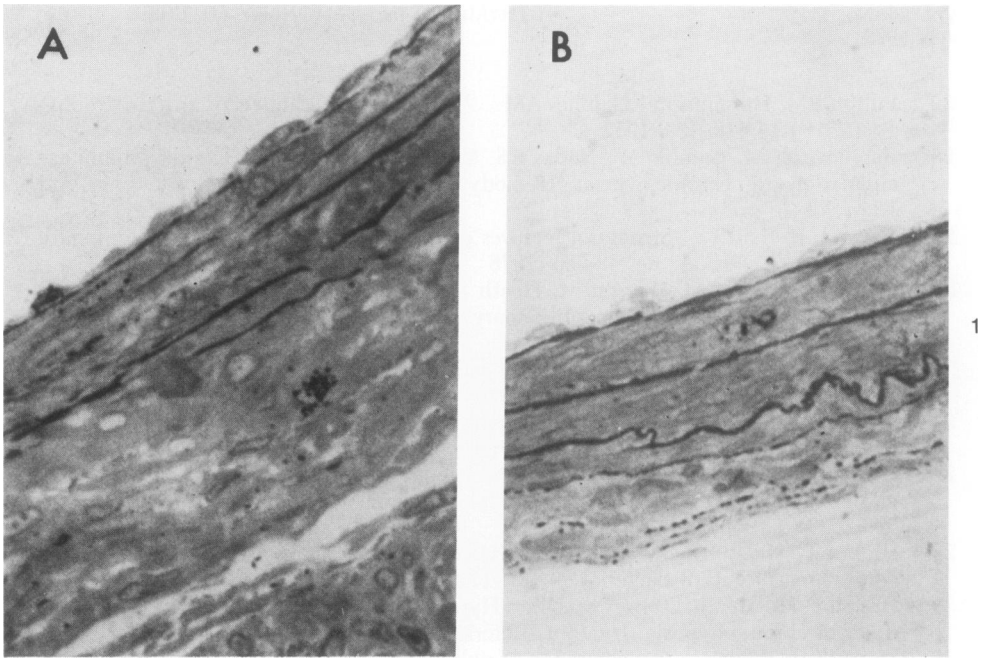

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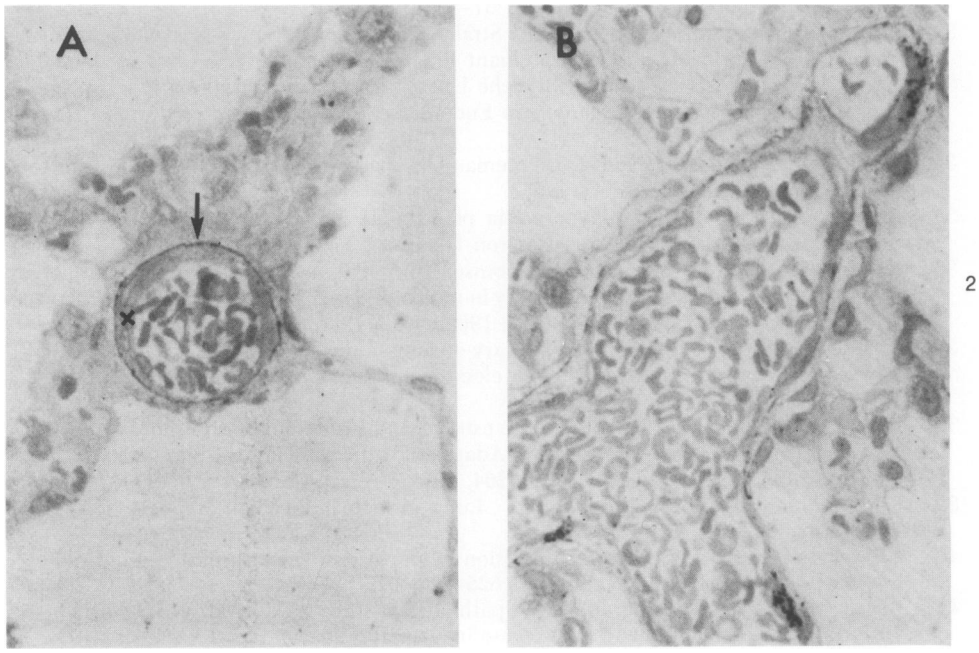

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Figures 1A and 1B—One-μm toluidine blue stained sections of the hilar muscular artery after 7 days' hypoxia showing **A)** a fibroblast and an endothelial cell and **B)** a smooth muscle cell labeled with ^{3}H-thymidine. (×600) **Figures 2A and 2B**—One-μm toluidine blue stained sections of **A)** a newly muscularized artery after 7 days' exposure to hypoxia. Note the single elastic lamina (↓) and new muscle cells (X), and **B)** a newly muscularized artery after 7 days' hypoxia with three labeled new or precursor smooth muscle cells in the wall. (×600)