Morphological Development of the Pulmonary Vascular Bed in Fetal Lambs

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SUMMARY The morphological development that accompanies increasing pulmonary blood flow and decreasing pulmonary vascular resistance with advancing gestational age has not been delineated. To study this point we developed a method of comparing pulmonary arterial vessels of the same generations in fetal lambs. Pulmonary arteries were perfused with glutaraldehyde solution at pressures appropriate for gestational age and then injected with an India ink-gelatin-Micropaque mixture. Using the dissecting microscope and serially prepared sections we studied successively smaller generations of arteries. We assessed the medial width/external diameter ratio in a total of 825 vessels from six fetal lambs of 85 to 140 days gestation. In fifth generation, or resistance vessels, this ratio remained constant over the gestational period studied (N = 529, Ȳ = 0.16, slope = 0.0003). We measured the volume of 17-34 randomly selected sections from each of the six fetuses, counted the total number of fifth and sixth generation vessels in these sections, and calculated the total number of these vessels per right lung. This number increased from 0.10 × 10⁶ to 4.08 × 10⁶ with increasing gestational age. The number of vessels per unit volume increased from 7.2 × 10⁴/ml to 61.8 × 10⁴/ml of right lung over this gestational period. The results indicate that increased pulmonary blood flow and decreased pulmonary vascular resistance with advancing gestation are due to an increase in the total number of vessels and increased vasomotor reactivity is related to an increase in the total amount of smooth muscle while the thickness of muscle in individual vessels remains constant.

☐ WITH INCREASING GESTATIONAL AGE in fetal lambs the pulmonary vascular bed becomes more responsive to vasoactive stimuli such as hypoxia. Based on previous studies of human fetal lungs this increased reactivity has been explained by an increasing amount of smooth muscle in small pulmonary arteries. However, during this same period of gestation, pulmonary blood flow increases and pulmonary vascular resistance decreases in the fetal lamb. It is difficult to correlate the morphologic finding of an increase in smooth muscle in individual pulmonary arteries with both the increase in pulmonary vasoreactivity and increase in pulmonary blood flow.

In contrast to those investigators who found an increase in the amount of pulmonary arterial smooth muscle with increasing gestational age, Hislop and Reid concluded that late in human gestation, there was no change in the amount of smooth muscle in small pulmonary arteries. However, they did not explain how an increase in pulmonary blood flow could be accounted for as the fetus approached term. Three factors may explain the differences in these studies. First, lungs were not perfused in the earlier investigations and used a standard high perfusion pressure of 100 cm H₂O for all gestational ages. Both the lack of perfusion pressure and perfusion at excessive pressures would tend to distort the medial muscle wall. Second, the vessels were selected by their size or association with particular airways. These criteria have never been shown to provide a basis for comparing the same kinds of vessels in terms of their position in the branching patterns of the pulmonary vascular bed during different gestational periods. Third, quantitation of medial wall smooth muscle was performed by a different method in each case. The techniques are not comparable and there is some question regarding the validity of each method when related to the type of fixation and vessel selection employed.

In order to clarify the discrepancies in the results of these previous investigations and to delineate the morphologic basis for the increasing pulmonary vasomotor reactivity and increasing pulmonary blood flow during gestation we developed a method of comparing similar generations of pulmonary arteries in fetal lungs of various sizes and stages of gestational development. This method is based upon the order of vessels in the pulmonary vascular bed and is therefore independent of size of vessels or their association with airways.

Methods

Fixation of Lungs

Pregnant ewes were given 3 ml of 1% tetracaine hydrochloride intrathecally and 300 mg sodium pentobarbital intravenously and an hysterotomy was performed. The fetus was given 1000 units of sodium heparin via the umbilical vein and delivered with its head in a fluid filled bag. The heart and lungs were dissected free within 2 to 3 minutes of delivery, a cannula passed into the main pulmonary trunk just distal to the pulmonic valve (preductal in the lamb) and a second cannula placed into the trachea. The ductus arteriosus was tied, and in order to clear blood from the vascular space, the lungs were perfused with 500 to 750 ml of 0.9% NaCl solution at a mean pressure appropriate for gestational age. Fetuses were grouped by age into 80-110
days, 111–130 days, and 131 days to term (term is 145 to 150 days in the sheep) and 30 mm Hg, 35 mm Hg, and 40 mm Hg of perfusion pressure, respectively, was used. A pressure of 10 cm H₂O was applied to the airway.

Immediately prior to each perfusion a glutaraldehyde fixative was prepared. Buffer consisted of 700 ml of distilled water, 9.6 g of sodium cacodylate powder and 67 g of polyvinylpyrrolidinone K-30 (PVP). This was filtered and adjusted to pH 7.5 with 9N NaOH. Preservative was made by diluting 50 ml of 50% glutaraldehyde with 75 ml of distilled water.

We prepared fixative A by adding 37.5 ml of the diluted glutaraldehyde to 210 ml of buffer and 1.8 ml of 30% hydrogen peroxide. This was adjusted to pH 7.5 with 9N NaOH. We prepared fixative B by adding 75 ml of the diluted glutaraldehyde to 420 ml of buffer and adjusting to pH 8 with 9N NaOH. We perfused the lung with fixative A and fixative B in succession at the appropriate pressures and applied 10 cm H₂O pressure to the airway with the fixative.

We prepared an India ink-†-gelatin-Micropaque‡ mixture in the following manner. After heating 70 ml of distilled water to 80°C, 10 g of gelatin, 0.25 g of thymol, 80 g of Micropaque, and 30 ml of biological grade India ink were successively stirred into the mixture.

After fixation we placed the lungs into a 0.9% NaCl solution bath at 40°C and gently irrigated the pulmonary vessels with NaCl solution at 40°C. This cleared the vascular space of fixative and warmed the lungs sufficiently to allow perfusion with the gelatin mixture. Only 1 to 5 ml of the mixture were needed to fill the pulmonary arterial bed. The mixture was viscous and therefore remained in the precapillary vessels. We then closed the arterial cannula and placed the lungs in fixative B in the refrigerator and allowed the gelatin to set overnight. A radiograph of the lungs was obtained to insure adequate filling of the pulmonary arterial tree.

Cutting the Lungs

We opened the right main stem bronchus for the length of the lung exposing the lumina of several airway branches (fig. 1). With a blade, a section of lung approximately 1 cm thick was cut, including an airway lumen, perpendicularly to the right main stem bronchus and extending to the periphery of the lung. We obtained four such specimens from each right lung of six fetuses from 85 to 140 days gestation.

The specimens were dehydrated in successive concentrations of 50%, 70%, 80%, 95% and 100% alcohol for one hour each. The dehydration in 100% alcohol was repeated twice and the specimens were stored overnight in 100%
alcohol. They were then placed in xylene for four hours. There was an average of 10% shrinkage during these steps as measured by fluid displacement for six specimens.

We defined the main pulmonary artery (a division of the main pulmonary trunk in the fetal lamb) as the first generation and the right pulmonary artery as the second (fig. 2). When a division which was at least 50% smaller than the second generation arose from it, we called this a third generation. Under the dissecting microscope, the pulmonary arterial system, filled with India ink-gelatin-Micropaque mixture, was visible in the cleared specimens (fig. 3). The right pulmonary artery traversed the thickness of the specimens in close association with the right main stem bronchus. When a division which was at least 50% smaller than the right pulmonary artery was seen arising directly from it, the vessel was cut across and a smaller block of tissue prepared to include this vessel. Usually one or two smaller blocks could be prepared from each large block of tissue.

The smaller blocks were infiltrated and embedded in paraffin at 60° C. Sections were prepared at 7µ, serially mounted, numbered and stained with iron hematoxylin and Van Geison's solution.9

The first large vessel on the first section of each serially prepared set of slides was a known generation, the third. We followed this using the light microscope until vessels which were at least 50% smaller arose from it and we called these fourth generation vessels. Fifth generation vessels were at least 50% smaller than fourth, and sixth generation vessels at least 25% smaller than fifth. After the sixth generation, we consistently found nonmuscular vessels in all lungs studied. When a vessel split into equally sized vessels we called this a branching order, not a new generation. For example, a third generation vessel may branch into two smaller third generation vessels. This type of branching was considerably less common than division into new generations of vessels.

**Measurements**

Third, fourth, fifth, and sixth generation vessels were photographed using high resolution color photomicrography film.* A calibrated 5/100 parts linear reticle was incorporated into the camera lens so that it appeared in each photomicrograph. Only vessels which could be clearly followed in serial sections were included. All vessels which could be identified, which were cut in cross section, and which were suitable for photography, were included in the study. Using the section numbering system great care was taken not to photograph the same vessel more than once.

We projected the individual photographic slides onto a screen (fig. 4) and used a fine caliper to measure the external diameter (d1) between the outer margins of the media (m), the internal diameter (d2) between the external borders of the intima (i) and the media between the external border of the intima and the external border of the media. We repeated these measurements at a 90° angle to the first measurements. The difference between the mean of the two external diameter measurements and the smallest of the two external diameter measurements for 20 fifth generation vessels selected at random from each of the six lungs (N = 120) was 1.68µ. The difference between these two measurements is not significant by unpaired t-test. After correction for magnification we calculated the mean external diameter, the mean medial width, and the mean medial width/mean external diameter for each of the 825 vessels photographed.

Under high power magnification (250× to 400×), all the fifth and sixth generation vessels (of any shape) in 17 to 34 randomly selected sections from each set of slides from each fetus were counted. Since the slides were serially mounted it was unlikely vessels in the same area of lung were counted more than once. Using the camera lucida the area of lung in which these vessels appeared was planimetered, multiplied by the thickness of the section (7µ), and corrected for 10% dehydration shrinkage. The mean number of fifth and sixth generation vessels per volume of lung prior to dehydration was calculated. The volume of the right lung after fixation was measured by fluid displacement and the total number of fifth and sixth generation vessels in each right lung was calculated.

* Kodak PCF 135–36 Number 2483.
Results

Medial Width (table 1)

Medial width progressively decreased from third to the sixth generation vessels at each gestational age. Although there was a great deal of variation in the few third generation vessels available for measurement, for the other generations the mean medial width within any one generation was remarkably similar throughout the period of gestation studied (fig. 5).

External Diameter (table 2)

Although the criteria for selection of vessels are such that the vessels in one axial pathway must get smaller from third through sixth generations, it is important that when all axial pathways are grouped, the mean external diameter also gets smaller from third through sixth generations in all animals.

For each fetus the third and fourth generations were compared by unpaired t-test, the fourth and fifth generations by Mann-Whitney test, and the fifth and sixth generations by unpaired t-test. All these comparisons were significant at the $P < 0.001$ level. Except for the third generation the mean external diameters were remarkably similar within any one generation throughout the period of gestation studied (fig. 6).

Medial Width/External Diameter (table 3)

The mean ratio of medial width divided by external diameter relates the amount of smooth muscle to the size of vessel. There was a progressive increase in this ratio from third to fifth generation vessels. Between the fourth and fifth generation vessels there was a sudden large increase in the amount of medial wall smooth muscle for the size of vessel (fig. 7). The difference between the fourth and fifth genera-
The 529 fifth generation vessels in the six fetuses studied all showed similar medial width/external diameter ratios (fig. 8). We determined the linear regression for these fifth generation vessels plotting medial width/external diameter versus gestational age. The slope of the line is 0.0003 (Y = 0.16). In figure 9 a line drawn through the mean values for fifth generation vessels at each gestational age depicts the similarity of the ratio at all gestational ages studied.

Total Number of Vessels

Although the amount of medial wall smooth muscle in individual resistance vessels is the same throughout the period of gestation studied, the number of vessels per right lung increased. In figure 10 we see the total number of fifth and sixth generation vessels per right lung increases from 0.10 × 10⁶ at 85 days to 4.08 × 10⁶ at 140 days gestation. The number of vessels increased from 7.2 × 10⁵/ml to 61.8 × 10⁵/ml of right lung over this gestational period. This is dramatically demonstrated by methylmethacrylate casts, figure 11A, of the pulmonary arterial bed of a 105-day (0.65 gestation) and 11B, of a 140-day (0.95 gestation) fetus. Vessels as small as 20μ external diameter are filled. There is an impressive increase in the number of small pulmonary arteries in the fetus near term.

To verify the importance of perfusing the pulmonary arterial bed, we fixed one lung from a fetus by perfusion and the other lung by diffusion. We measured 15 fifth generation vessels from each lung and the medial wall thickness was 6.25μ (sd = 1.39μ) in the perfused lung and 7.42μ (sd = 1.64μ) in the lung fixed by diffusion (P < 0.05 by unpaired t-test). We also calculated the area of the cross section of the media [Acsm = 0.785 (d₁² - d₂²)] in the vessels of the two lungs prepared by perfusion versus diffusion. In the perfused lung this area was 514.18μ² (N = 15, sd = 172.69μ²) and in the nonperfused lung fixed by diffusion it was 704.59μ² (N = 15, sd = 276.08μ²) (P <0.05 by unpaired t-test).

Discussion

There has been a great variation in the methods used for fixation, selection of vessels, and quantitation of pulmonary arterial smooth muscle (table 4). Wagenvoort,¹¹ in a study of children with congenital heart disease, demonstrated that the media of pulmonary arteries in nonperfused lungs was on the average 7.8% thicker than in perfused lungs; we have confirmed this finding. However, neither he nor other investigators,²,³ perfused the lungs when studying the fetus. Hislop and Reid⁴ did perfuse lungs but used a standard pressure of 100 cm H₂O. Fixation of vessels at a perfusion pressure higher than that present during life could have resulted in excessive distension and the vessel wall could be unduly thinned. Although it is not possible to reproduce ex-

### Table 1. Medial Width

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Third</th>
<th>Fourth</th>
<th>Fifth</th>
<th>Sixth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Media (μ)</td>
<td>N Media (μ)</td>
<td>N Media (μ)</td>
<td>N Media (μ)</td>
</tr>
<tr>
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<td>4</td>
<td>31.67 (15.77)</td>
<td>20</td>
<td>8.82 (4.33)</td>
</tr>
<tr>
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<td>2</td>
<td>20.25 (9.55)</td>
<td>14</td>
<td>8.53 (4.07)</td>
</tr>
<tr>
<td>120</td>
<td>1</td>
<td>83.25 (3.30)</td>
<td>11</td>
<td>8.09 (2.57)</td>
</tr>
<tr>
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<td>2</td>
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<td>18</td>
<td>8.50 (3.30)</td>
</tr>
<tr>
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<td>5</td>
<td>21.62 (8.61)</td>
<td>23</td>
<td>7.96 (2.84)</td>
</tr>
<tr>
<td>140</td>
<td>3</td>
<td>63.62 (17.31)</td>
<td>18</td>
<td>14.15 (8.57)</td>
</tr>
</tbody>
</table>

μ = microns; N = number; ( ) = 1 standard deviation.

### Table 2. External Diameter

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Third</th>
<th>Fourth</th>
<th>Fifth</th>
<th>Sixth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Diameter (μ)</td>
<td>N Diameter (μ)</td>
<td>N Diameter (μ)</td>
<td>N Diameter (μ)</td>
</tr>
<tr>
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<td>4</td>
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<td>20</td>
<td>177.6 (129.2)</td>
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<tr>
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<td>2</td>
<td>732.0 (169.7)</td>
<td>14</td>
<td>205.3 (123.0)</td>
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<tr>
<td>120</td>
<td>1</td>
<td>1656.0 (89.1)</td>
<td>11</td>
<td>197.4 (137.1)</td>
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<tr>
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<td>1455.0 (247.2)</td>
<td>18</td>
<td>214.5 (121.3)</td>
</tr>
<tr>
<td>135</td>
<td>5</td>
<td>672.6 (247.2)</td>
<td>23</td>
<td>144.5 (121.3)</td>
</tr>
<tr>
<td>140</td>
<td>3</td>
<td>1196.0 (234.6)</td>
<td>18</td>
<td>284.8 (196.0)</td>
</tr>
</tbody>
</table>

μ = microns; N = number; ( ) = 1 standard deviation.
actually the conditions during life, it would seem important to try to maintain vessel size as close as possible by perfusing the pulmonary arterial bed of each lung at appropriate pressure.

O'Neal et al.\(^3\) selected vessels which were associated with bronchioles and linked small muscular arteries to capillaries. Bucher and Reid\(^7\) have demonstrated that identifying features of airways change with the progression of gestation and therefore selection of vessels in relation to specific airway generation may lead to comparison of different vessels at different gestational ages. For example, the number of generations of airways which were fully epithelialized diminished after the twentieth week of gestation due to the ingrowth of capillaries. Therefore airways which at an earlier stage of gestation would be identified as bronchioles, at a later stage become respiratory bronchioles. Wagenvoort et al.\(^4\) apparently assumed that resistance vessels are smaller in smaller fetuses and therefore selected smaller vessels (\(< 70 \mu\), or \(< 50 \mu\), or \(< 40 \mu\)) as they studied smaller fetuses. Our data show that this would result in the selection of different generation of vessels at each stage of gestation and therefore make comparison difficult. The resistance vessels do not change in size during the last half of gestation in the fetal lamb. Naeye\(^6\) selected vessels of less than 50\(\mu\), and Hislop and Reid\(^6\) classified vessels by their observed size. These criteria do not consider whether or not vessels of similar type are being compared at different stages of gestation.

We wished to study vessels during different stages of gestation in relation to their order in the pulmonary vascular bed rather than on the basis of size or airway association. Von Hayek\(^8\) and Nagaishi\(^9\) provide evidence to support the concept that successively smaller generations of vessels rather than classical dichotomous branching is the pattern of division within the pulmonary arterial bed. Von Hayek says "The divisions of the pulmonary arteries are bifurcations as a rule. The two branches vary widely in size. In this they follow the rule which is well known for the systemic circulation, that the larger branch shows the smaller deviation from the direction of the stem." The divisions of the pulmonary artery in the lamb are not dichotomous but a series of successively smaller vessels. Many vessels of the next smaller generation arise from one axial vessel.

Great variation in the size of the third and to a lesser extent the fourth generation vessels may be due to fewer

![Figure 7](http://circ.ahajournals.org/)

**Figure 7** Comparison of medial width/external diameter ratio of different generations. Note the sudden increase in the ratio between the fourth and fifth generations.
numbers available for analysis but is more likely due to functional demand in relation to the volume of lung which they supply in different size fetuses and in different areas of the same lung. This study demonstrates that fifth and sixth generation vessels are similar in size in lungs of various stages of gestational development and throughout the same lung. This may be due to the smaller peripheral area of lung supplied by each vessel, which is similar at any of the later stages of development.

In order to quantitate the amount of smooth muscle in pulmonary arteries, O'Neal et al.² used a relative ranking system (0–3) which presents difficulties in terms of comparability and reproducibility. Wagenvoort et al.⁴ calculated the area of the cross section of the media based upon linear measurement of media and external diameter and divided this by the area of lung in which the vessels appeared. Our calculations of cross sectional area of the media in a perfused versus nonperfused lung show that since Wagenvoort and his associates did not perfuse the fetal lungs, there would be an overestimation of the amount of smooth muscle as determined by their method.

Naeye⁵ planimetrered the area of the media and divided this by the area of the intima, arguing that the area of the intima is almost constant throughout the gestational period studied and that as the resistance vessels grow late in gestation the intimal area of the new resistance vessels is the same at any given size. Substantiation of this technique has been lacking. Hislop and Reid⁶ used the ratio of two times the wall thickness divided by the external diameter multiplied by 100% to quantitate pulmonary arterial smooth muscle. Our method of measurement of one medial wall thickness divided by the external diameter is a variation of this and in properly perfused lungs is a reliable means of assessing the amount of smooth muscle present in any size artery.

Although there is no specific comment in the literature, the results of previous investigations support our finding of an increase in the number of resistance vessels with increasing gestational age. O'Neal et al.⁴ added the relative rank (0–3) of pulmonary arterial muscle to the relative rank (0–3) of number of arteries seen and the arithmetical mean of these two ranks yielded an index of smooth muscle which increased with increasing gestation. Wagenvoort et al.⁴ multiplied the mean area of the cross section of the media by the calculated total numbers of small pulmonary arteries to

![Figure 8](http://circ.ahajournals.org/)

**Figure 8** Fifth generation vessels from A) an 85-day fetus (m/d = 0.17), B) a 105-day fetus (m/d = 0.16), C) a 122-day fetus (m/d = 0.16), and D) a 140-day fetus (m/d = 0.17). All vessels are magnified 400X and each small division of the reticle represents 1.5μ.

![Figure 9](http://circ.ahajournals.org/)

**Figure 9** Mean values for medial width/external diameter ratio for all fifth generation vessels from the six fetuses. N = number of fifth generation vessels. The brackets indicate plus and minus 1 standard deviation.

![Figure 10](http://circ.ahajournals.org/)

**Figure 10** Total number of fifth and sixth generation vessels per right lung for each fetus. The line shows a nearly linear increase with increasing gestational age.
derive their index, which also increased with increasing gestational age. Hislop and Reid\(^4\) found the ratio of wall thickness to external diameter unchanged in small vessels (<150\(\mu\)) after 16 weeks gestation and an increase in the number of supernumerary vessels from 12 to 36 weeks. Naeye\(^6\) found an increase in the amount of smooth muscle in individual pulmonary arteries with increasing gestation and this may be explained by the use of the intimal area which shows wide variation as an internal baseline.

Although previous investigators have not emphasized the finding of an increase in the total number of small pulmonary arteries we feel this is the key to understanding the paradox of an increase in pulmonary vasomotor reactivity and increased pulmonary blood flow with increasing gestational age. The increase in the total number of vessels rather than an increase in muscle in each vessel explains the increased pulmonary vasoreactivity with increasing gestational age. At the same time the increasing number of vessels increases the cross sectional area of the pulmonary vascular bed thus explaining the increase in pulmonary blood flow with increasing gestational age. The stimulus for the development of new vessels is unknown but could be related to low level of blood oxygen perfusing the pulmonary arterial bed in the fetus.

Although there may be some species difference for absolute values of medial width/external diameter ratio and the total number of vessels per right lung volume, inspection of pulmonary arterial casts and angiograms from human fetuses have encouraged us to utilize these techniques for studying the morphological development of the pulmonary vascular bed in human fetuses and newborns.

Acknowledgments

We gratefully acknowledge the advice and assistance of Dr. James Robert Hennessey, Dr. Julien J. E. Hoffman and Donald McDonald, and the technical assistance of Lutheran Dong, Louise Wong and Alan Nisen.

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Morphological development of the pulmonary vascular bed in fetal lambs.
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Circulation. 1976;53:144-151
doi: 10.1161/01.CIR.53.1.144
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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