Thickness of the blood-gas barrier in premature and 1-day-old newborn rabbit lungs

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Fu, Zhenxing, Gregory P. Heldt, and John B. West. Thickness of the blood-gas barrier in premature and 1-day-old newborn rabbit lungs. Am J Physiol Lung Cell Mol Physiol 285: L130–L136, 2003. First published March 14, 2003; 10.1152/ajplung.00366.2002.—The pulmonary capillaries of neonatal lungs are potentially vulnerable to stress failure because of the complex changes in the pulmonary circulation that occur at birth. We studied the ultrastructure of the blood-gas barrier (BGB) in premature and 1-day-old rabbit lungs and compared it with the ultrastructure of adult lungs. Normal gestation of rabbits is 30 days. After extensive pilot measurements, three premature (27 days gestation) and three newborn (1 day old) rabbit lungs were perfusion-fixed at arterial, venous, and airway pressures of 25, 0, and 10 cmH2O, respectively, and the measurements were compared with those of three adult lungs. The thickness of the capillary endothelium, alveolar epithelium, and interstitium of the BGB was measured at right angles to the barrier at random points. A striking finding was the large number of measurements of the interstitial thickness in 1-day-old lungs that were very thin (0–0.1 μm). The percentages of occurrence of very thin interstitium in premature, 1-day-old, and adult lungs were 35.3 ± 9.4, 71.7 ± 5.2, and 43.0 ± 2.6, respectively (P < 0.02 for 1 day old vs. premature and adult). Given the previously found relationship between stress failure and interstitial thickness, this large proportion of very thin interstitial layers in the capillaries of 1-day-old lungs is a reasonable explanation for their previously demonstrated vulnerability to stress failure.

lung morphology; capillary stress; stress failure; pulmonary edema; pulmonary hemorrhage

WHEN THE PULMONARY CAPILLARIES of adult lungs are exposed to transmural pressures that exceed the normal maximal physiological values, ultrastructural changes occur in the walls, a condition known as stress failure (20, 23). A common result is the development of a high-permeability form of pulmonary edema with high-molecular-weight proteins and red blood cells in the alveolar fluid.

The pulmonary capillaries of neonatal lungs are potentially vulnerable to stress failure because of the complex changes in the pulmonary circulation that occur at birth. We have previously shown that the capillaries of newborn rabbit lungs are more fragile than those in the adult on the basis of the capillary transmural pressure required to cause stress failure (9). In the fetus, the pulmonary arterial pressure is approximately the same as the systemic arterial pressure because of the large patent ductus arteriosus. The magnitude of the pressure, ~50 mmHg in the fetal lamb (18), is more than enough to cause ultrastructural damage to pulmonary capillaries in the adult rabbit (20). Fortunately, the fetal capillaries are protected from the high pressure by the constriction of pulmonary arterioles, which is made possible by the large amount of vascular smooth muscle in the fetal lung and by the high pulmonary artery vascular tone.

At birth, a complex series of events occurs. Pulmonary blood flow rapidly increases from ~15% of the cardiac output before birth to 100% of the cardiac output. This is accomplished by a dramatic reduction in pulmonary arteriolar vasoconstriction brought about in part by the relief of hypoxic vasoconstriction and also by inflation of the lung. This large and rapid reduction in vasoconstriction would ordinarily greatly increase the transmural pressure of the pulmonary capillaries. However, the pulmonary arterial pressure is simultaneously reduced by narrowing of the ductus arteriosus in the minutes and hours after birth.

It is easy to imagine that if the fall in pulmonary arterial pressure does not accompany the release of pulmonary vasoconstriction, the pulmonary capillaries will be exposed to an increased transmural pressure. The resultant stress failure would cause leakage of plasma and red blood cells into the alveolar spaces. The effect of the plasma in the alveoli would be to inhibit pulmonary surfactant (19) and potentially to contribute to respiratory failure.

Although several studies have addressed the development of the lung from the fetal to the adult state (5–7, 13), little attention has been specifically focused on the components of the blood-gas barrier (BGB) in the term newborn and premature lung. We are not aware of any morphometric analyses that would help us to understand the relative strength of the BGB in the term newborn and premature lungs compared with the adult.

The purpose of the present study was to carry out a morphometrical analysis of the BGB in premature, term 1-day-old, and adult rabbit lungs. The emphasis is on those features of the barrier that would make the
capillaries vulnerable to stress failure in the event that they are exposed to a high capillary transmural pressure. The methods were the same as those previously used to relate the morphometry of the BGB to the strength of the capillaries in adult rabbit, dog, and horse (2) where our laboratory showed that the thinner the interstitium, the more fragile are the capillaries.

MATERIALS AND METHODS

Animals

The protocols for the present study were approved by the Animal Subjects Committee at the University of California, San Diego. Care and the handling of rabbits was according to the guidelines of the National Institutes of Health.

The small size and the fragility of neonatal lungs, and especially those from premature animals, made it technically very difficult to cannulate the pulmonary artery and left atrium to carry out the perfusion fixation in situ at known vascular and airway pressures. Many preliminary studies were carried out, and the results from nine New Zealand White rabbits from three different age groups are reported here: three premature (27th day of gestation) rabbit pups from two mothers, three term newborn (1 day old) rabbit pups from one mother, and three adult rabbits.

Anesthesia and Surgery

Premature pups. Premature pups were obtained from a dated-pregnant rabbit on the 27th day of gestation. The breeding day was considered day 0, and full term is 30 days. The pregnant rabbit was initially anesthetized with a mixture of ketamine (25 mg/kg) and xylazine (8 mg/kg) by intramuscular route and was maintained thereafter by intravenous pentobarbital sodium (10–15 mg/kg) given as needed approximately every 45 min. Cesarean section was accomplished through a midline incision and a 2- to 3-cm uterotomy. The fetuses were delivered from the uterus one at a time and given norcurin (1 mg/kg) and pentobarbital sodium (10–15 mg/kg).

A tracheostomy tube was placed in each rabbit pup through a midline incision of the trachea. An 18-gauge blunt needle was used for the tracheal cannula. The pup was then placed in a heated plethysmograph and ventilated for 5 min with oxygen with a respiratory rate of 30 breaths/min, peak inspiratory pressure of 25 cmH2O, and no end-expiratory pressure. This provided uniform lung inflation and initial resuscitation. The pup was then removed from the plethysmograph, and the tracheostomy was attached to a water manometer to maintain an airway pressure of 10 cmH2O. A midline thoracotomy was performed. The heart was exposed, and 10 units of heparin were injected into the right ventricle. A small vascular clamp was placed on the ductus arteriosus. Two 24-gauge Angiocaths were separately inserted into the right and left ventricles and advanced into the main pulmonary artery and the left atrium. The left atrial catheter was advanced through a midline incision and a 2- to 3-cm uterotomy. The left atrial catheter was then deployed in the pulmonary artery and kept at that pressure for the duration of the perfusion fixation. This alveolar pressure was required to keep the lungs uniformly inflated. The pulmonary vascular pressure was perfused via the pulmonary artery with a saline-dextran mixture (11.06 g/l NaCl, 350 mosM; 3% T-70 dextran; 10 IU/ml heparin), which continued for ~2–3 min to wash out the blood. The lungs were then fixed by perfusion for 10 min with buffered glutaraldehyde solution (phosphate-buffered 2.5% glutaraldehyde with 3% T-70 dextran; 500 mosM, pH 7.4). For all perfusions, the pulmonary arterial pressure was set at 25 ± 1 cmH2O, using reservoirs maintained at a fixed height above the preparation while the pulmonary venous pressure was atmospheric pressure (0 cmH2O). There are published data available on the pulmonary arterial pressure in neonatal lambs (3, 10, 11) but not rabbits. We assumed that the pulmonary arterial pressure in the neonatal rabbit is close to that of the lamb (~20 mmHg).

Three adult rabbits were used. They were prepared for in situ perfusion fixation as previously described using pulmonary artery, alveolar, and venous pressures of 20, 5, and 15 cmH2O, respectively (20). Although we attempted to use an alveolar pressure of 5 cmH2O, we found that this was insufficient to keep the lungs expanded. We therefore used a pressure of 10 cmH2O, and this was just enough to give uniform expansion.

Tissue Sampling and Preparation

The lungs were removed after the fixation and stored in buffered glutaraldehyde at 4°C. A single transverse slice, ~0.3 cm thick, was cut from the left caudal lobe of each lung at about one-third the distance from the most caudal aspect. A vertical sampling procedure was used to systematically select tissue blocks from a vertical slice from each slab (12). The blocks were rinsed in 0.1 M phosphate buffer (350 mosM, pH 7.4), postfixed for 2 h in 1% osmium tetroxide in 0.125 M sodium cacodylate buffer (400 mosM, pH 7.4), dehydrated in increasing concentrations (70–100%) of ethanol, rinsed in propylene oxide, and embedded in Araldite. For light microscopy, ultrathin sections (50–70 nm) contrasted with uranyl acetate and bismuth oxinitrate (17) were examined with a Zeiss 10 electron microscope.

Morphometric Analysis

Light microscopy. For each rabbit lung, a total of 40 digitized light micrographs were taken by systematic random sampling from a single semithin section (1 μm) from each of 2 blocks. All micrographs were saved in an IBM computer using the Photoshop program. A computer-generated test grid containing 10 vertical and 10 horizontal lines was superimposed onto each micrograph. Each test grid has 100 test points (the intersection of test lines). Lung images were calibrated using a microscopy calibration grid with one pixel on the computer monitor equal to 0.3 μm. We chose to designate all the BGBs thinner than 0.6 μm (2 pixels) as "thin" because the average thickness of the thin side of the BGB in the adult rabbit is 0.55 μm (2). All the BGBs on which a test point fell were recorded by a manual counter as one of three groups: thin BGB (~0.6 μm, or 2 pixels), thick BGB (0.6–1.5 μm, or 5 pixels) or non-BGB (~1.6 μm or the portion of capillary wall not facing airways). In all cases, the linear measurement was made at right angles to the BGB. The fractions of thin, thick, and non-BGB were determined by this method.
Electron microscopy. For each rabbit lung, a total of 60 electron micrographs were taken (30 micrographs taken by systematic random sampling from a single ultrathin section from each of 2 blocks). A micrograph of a carbon grating replica (E. F. Fullam, Schenectady, NY) was taken on each film for calibration.

All the negatives of electron micrographs were scanned and saved on a compact disk. An IBM personal computer loaded with Matlab 5.3 was used to determine the thickness of the endothelial, epithelial, and interstitial layers of BGB. Interstitium refers to the tissue between the epithelial and endothelial layers. All measurements were made at right angles to the BGB. Prints of each negative were available for positive identification of structures on the computer screen, as needed, during measurement. The sites for measuring the thickness of the various layers of the BGB were determined at random by intersection of the barrier with fixed test lines generated by the program (up to 5 intersections per micrograph).

Statistics

Data on individual animals are expressed as means ± SD. The mean values for each age group are expressed as means ± SE. Group means were compared by one-way ANOVA with appropriate post hoc testing. Differences were taken as significant for *P* < 0.05.

RESULTS

**Morphology**

Premature lung. The light and electron microscopic images (Fig. 1, A–C) show that the parenchyma of premature rabbit lungs appear very cellular in contrast to those of adults. The septa are thicker and mostly possess capillaries on both sides, which is a sign of immaturity of lung parenchyma. There are many fibroblasts and other types of interstitial cells between the two capillary layers, especially in regions where several septa join (Fig. 1A). Electron micrographs reveal that both the endothelium and epithelium are thick and often have many mitochondria in their cytoplasm (Fig. 1C). It is rare to see the secretion products of lamellar bodies from type II cells within airways.

Fig. 1. Light and electron micrographs of portions of lung parenchyma in premature (A–C) and term newborn (D–F) rabbits prepared by perfusion-fixation. Note the reduced amount of cellular interstitium and the thinner blood-gas barrier (BGB) in the term newborn (D and E) compared with the premature animal (A and B). C: fibrils of collagen (arrowhead) are fused together with the basement membrane in the thin side of the BGB in premature lungs. Also, there are many mitochondria in endothelium and epithelium (arrows). F: cytoplasmic projections (open arrows) of endothelial cells extended into the capillary lumen of lung parenchyma in newborns, and secretion products of lamellar bodies can also be seen. a, Alveolus; c, capillary.
Fibrils of collagens are often fused together with the basement membrane in the thin side of the BGB (arrowhead, Fig. 1C).

One-day-old lung. The appearance of parenchyma of the newborn lung (Fig. 1, D–F) is similar to that of the premature lung except that the septa are thinner and less cellular (Fig. 1D). The electron micrographs reveal that the endothelium and epithelium are much thinner compared with premature lungs and that there are fewer mitochondria in the cytoplasm (Fig. 1E). There are many cytoplasmic projections of endothelial cells into the lumen and secretion products of lamellar bodies can be often seen in the airways (arrowheads) (Fig. 1F).

Morphometry

Light microscopy. The mean fraction of the thin, thick, and non-BGB in the lung parenchyma of three age groups are given in Table 1 and Fig. 2. The premature lungs have the smallest frequency (6.3%) of the measurements of the capillary walls with a thickness of <0.6 μm. The largest portions of the walls (82.3%) are facing the space between the double capillary layers and would be very inefficient for gas exchange. The percentage of the BGB <0.6 μm in the 1-day-old has almost doubled to 11.7% compared with the premature animals, but it still remains very low compared with the adults (56.2%).

Electron microscopy: thickness of BGB components. Table 2 shows the measurements of the thickness of the endothelium, interstitium, and epithelium and the total thickness of the BGB in premature, 1-day-old, and adult rabbit lungs. Figure 3 shows the percentages of measurements, and Fig. 4 shows the cumulative values. It can be seen that the endothelium, epithelium, and total thickness were substantially greater in the premature lungs than in adult lungs. In addition, the means for endothelium, epithelium, and total thickness were greater in 1-day-old lungs than adult lungs, although the differences particularly for endothelium were not so great. However, the epithelium thickness was greater in 1-day-old than adult lungs. These larger thicknesses of the endothelium and particularly the epithelium in premature and 1-day-old animals are consistent with the known thinning of the epithelium and endothelium that occurs in late gestation and shortly after birth.

A feature of Figs. 3 and 4 is the large number of measurements of the interstitium that have much smaller values in the 1-day-old than either the premature or adult lungs. In Fig. 3, this is shown as the markedly leftward shift of the peak of the curve for interstitium in 1-day-old lungs. In Fig. 4, the cumulative plot for the interstitium of 1-day-old lungs falls to the left of the plots for premature and adult lungs. The quantitative differences are emphasized in Fig. 5, which shows that in the 1-day-old lungs, 71.7 ± 5.2% of the measurements of interstitial thickness were ≤0.1 μm, whereas the percentages for premature and adult lungs were only 35.3 ± 9.4 and 43.0 ± 2.6, respectively (P < 0.02 for 1 day old vs. premature and adult).

DISCUSSION

Thickness Of The Interstitium Of The BGB

The most important finding of this study is that a large proportion of measurements of the thickness of the interstitium show very small values in 1-day-old lungs (Figs. 3–5). There is evidence that the strength of the BGB is primarily determined by the interstitium, and previous studies from our laboratory on comparative aspects of the strength of the BGB in the rabbit,
dog, and horse have shown that the thinner the interstitium, the less capillary transmural pressure is required to cause stress failure (2). Therefore, the present study suggests that the capillaries of 1-day-old lungs are particularly vulnerable to damage if the capillary pressure rises.

Although the mean value of the thickness of the interstitium in 1-day-old lungs is less than the mean values for premature and adult lungs, the differences are not statistically significant. However, the vulnerability for stress failure depends on not only the mean value but also whether there is an unusually large number of parts of the BGB that have a very thin interstitium. Figures 3–5 show that this is the case, and therefore it is reasonable to conclude the 1-day-old lungs are particularly vulnerable to stress failure.

It might be thought that the fact that the alveoli are fluid filled before birth would increase the thickness of the interstitium of the BGB and therefore introduce an artifact. However, this is clearly not the case for 1-day-old lungs because the interstitium is substantially thinner than that in the adult. Indeed, insofar as fluid in the interstitium may influence our results, the interstitium of 1-day-old lungs in the absence of interstiti-

Table 2. Mean thickness of the 3 layers of the blood-gas barrier in premature, newborn, and adult rabbit lungs

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>n</th>
<th>Endothelium</th>
<th>Interstitium</th>
<th>Epithelium</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Premature</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>84</td>
<td>0.41 ± 0.28</td>
<td>0.41 ± 0.63</td>
<td>0.54 ± 0.44</td>
<td>1.37 ± 0.84</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>0.37 ± 0.28</td>
<td>0.24 ± 0.21</td>
<td>0.53 ± 0.42</td>
<td>1.15 ± 0.58</td>
</tr>
<tr>
<td>3</td>
<td>130</td>
<td>0.30 ± 0.17</td>
<td>0.13 ± 0.17</td>
<td>0.41 ± 0.33</td>
<td>0.84 ± 0.45</td>
</tr>
<tr>
<td>Group means ± SE</td>
<td></td>
<td>0.36 ± 0.03*</td>
<td>0.26 ± 0.08</td>
<td>0.49 ± 0.04*</td>
<td>1.12 ± 0.15*</td>
</tr>
<tr>
<td><strong>1 Day Old</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>241</td>
<td>0.24 ± 0.20</td>
<td>0.11 ± 0.11</td>
<td>0.27 ± 0.17</td>
<td>0.64 ± 0.31</td>
</tr>
<tr>
<td>2</td>
<td>220</td>
<td>0.21 ± 0.16</td>
<td>0.09 ± 0.09</td>
<td>0.26 ± 0.14</td>
<td>0.56 ± 0.26</td>
</tr>
<tr>
<td>3</td>
<td>234</td>
<td>0.25 ± 0.22</td>
<td>0.13 ± 0.23</td>
<td>0.29 ± 0.19</td>
<td>0.68 ± 0.39</td>
</tr>
<tr>
<td>Group means ± SE</td>
<td></td>
<td>0.23 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.27 ± 0.01†</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td><strong>Adult</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>232</td>
<td>0.19 ± 0.17</td>
<td>0.17 ± 0.31</td>
<td>0.18 ± 0.29</td>
<td>0.54 ± 0.55</td>
</tr>
<tr>
<td>2</td>
<td>246</td>
<td>0.17 ± 0.15</td>
<td>0.19 ± 0.20</td>
<td>0.18 ± 0.11</td>
<td>0.55 ± 0.37</td>
</tr>
<tr>
<td>3</td>
<td>236</td>
<td>0.18 ± 0.10</td>
<td>0.16 ± 0.17</td>
<td>0.19 ± 0.01</td>
<td>0.52 ± 0.28</td>
</tr>
<tr>
<td>Group means ± SE</td>
<td></td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.08</td>
<td>0.54 ± 0.01</td>
</tr>
</tbody>
</table>

Values for each animal are means ± SD; n, no. of measurements. *Significantly different from 1 day old and adult, P < 0.01. †Significantly different from adult, P < 0.05.
tial fluid must be thinner than shown, and therefore the capillaries are even more vulnerable to stress failure. It is possible that the thickness of the interstitium in premature lungs was influenced by fluid filling of the alveoli, but in fact the thickness of the interstitium is not very different from that in the adult. By contrast, the endothelial layer, and particularly the epithelial layer, were markedly thickened in the premature lungs compared with the adult (Figs. 2 and 3), and this explains the much greater thickness of the total BGB. As noted earlier, the much greater thickness of the epithelium and endothelium in premature lungs is consistent with the known thinning of these layers as lung development proceeds.

Implications for Vulnerability of the Capillaries

There is evidence that the interstitium and in particular the basement membranes are responsible for the ultimate strength of the BGB, at least on the thin side of the BGB (22). This is consistent with previously published work by our laboratory (20) on the pattern of the stress failure that is observed in the BGB of the adult rabbit. Breaks are typically seen in the endothelial and epithelial layers or both in the BGB, but the basement membranes are often continuous, at least in the plane of the section. This suggests that the extracellular matrix is the strongest part of the alveolar wall.

Other evidence comes from studies (21) of isolated renal tubules that were subjected to increased luminal pressure when attached to micropipettes. The walls of the tubules contain only epithelium and basement membrane, and it was possible to remove the epithelium with detergent. It was found that the mechanical properties of the tubule were the same irrespective of whether the epithelium was present or not and that their mechanical behavior depended only on the extracellular matrix.

On the other hand, Parker and associates (15, 16) have provided evidence that endothelial cells have a role in the permeability of microvessels at high transpulmonary pressures. More work is required to clarify the relative importance of the extracellular matrix and the endothelium.

Another major factor that could contribute to stress failure of pulmonary capillaries in the newborn lung is mechanical ventilation at high lung volume. Fu et al. (8) showed that inflating the adult lung to high volumes greatly increased the number of disruptions in both capillary endothelial and alveolar epithelial cells. Newborn lungs, especially in premature infants, are prone to develop atelectasis, and mechanical ventilation is frequently the treatment of choice. Even if the total volume of the lung is not abnormally high as a result, some regions may be overexpanded, resulting in localized stress failure of pulmonary capillaries.
There is evidence that the pulmonary capillary bed of postnatal mammals such as rats and rabbits is changing rapidly once extrauterine life starts. We found in the present study that many of the capillary endothelial cells in newborn lungs have many cytoplasmic projections into the capillary lumen (Fig. 1F), which represent the characteristics of active migration of endothelial cells and the formation of new capillaries (angiogenesis) (1). Brody and associates (4) found in newborn rats that the basement membrane in the alveolar walls does not continuously cover all the endothelial cells of pulmonary capillaries. Only about one-third of pulmonary capillaries had endothelial cells that were 100% covered by a continuous basement membrane, one-third of the capillaries had 90–99% of the endothelial cells covered, and one-third had 70–90% covered, compared with 100% coverage of the endothelial cells in adults. Also, the study of mechanical properties of rat lung during postnatal development suggested that the lung elastic recoil is low and that lungs rupture at a lower transpulmonary pressures than that in the adult (14). All these studies suggest that newborn lungs are undergoing extensive structural changes at birth. Furthermore, the continual disruption and reforming of the cell-extracellular matrix bonds and cell-cell conjugations taking place may make the BGB vulnerable to increased stresses in the capillary wall.

In conclusion, the present study reports that random measurements of the thickness of the interstitium of the pulmonary capillary wall show that parts of this are remarkably thin in 1-day-old lungs compared with either premature or adult animals. The implication is that the BGB in 1-day-old lungs has a reduced mechanical strength despite the potential hazards posed by the changes in vascular pressures at birth. This is further evidence of the fragility of capillaries in newborn lungs in addition to that previously derived from their responses to increased transmural pressure (9). If stress failure of the pulmonary capillaries takes place, plasma and red blood cells will leak into the alveolar spaces with consequent inhibition of surfactant. This surfactant inhibition could contribute to the respiratory failure seen in the classic infant respiratory distress syndrome.

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