Changes in alveolar septal border lengths with postnatal lung growth

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Wood, Jonathan P., John E. Kolassa, and John T. McBride. Changes in alveolar septal border lengths with postnatal lung growth. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L1157–L1163, 1998.—Evaluation of alveolar development beyond the postnatal period of rapid septation has generally involved alveolar counting. We used an alternate approach to assess postseptation parenchymal development: measurement of the lengths of various types of alveolar septal borders. This technique directly addresses changes in the elastin fiber network that determines parenchymal complexity. Lungs from weanling and adult ferrets, inflated to 15 cmH2O, were perfused fixed and dehydrated, and 2-μm sections were stained with Miller's elastin stain for light microscopy. We used standard morphometric methods to measure the lengths of the various types of alveolar septal borders. Three types comprised >90% of all septal borders: 1) free septal ends ("ends") containing an elastin cable; 2) angled meetings of two alveolar septa ("bends"), also with a cable; and 3) the near-symmetrical intersections of three septa ("junctions") devoid of elastin. When scaled for lung volume, ends and bends were 23 and 37% greater in adults (P < 0.001), reflecting the increase in parenchymal complexity with growth. The 17% difference in scaled junction lengths was not significant (P = 0.10). Bends increased out of proportion to the increase in ends, and both bends and ends increased to a greater degree than any possible increase in junctions (P < 0.001 for all comparisons). Although the interpretation of changes in the distribution of alveolar border lengths is not straightforward, an increase in bends resulting in an increase in the complexity of individual alveoli may contribute to the increase in alveolar gas-exchanging surface area with growth. Septation, the process responsible for the rapid early postnatal increase in parenchymal complexity in many species, should tend to increase the lengths of ends and junctions and decrease the lengths of bends. Therefore, these data suggest that septation is not the predominant mechanism of later postnatal parenchymal development in the ferret.

alveolar growth; alveolar development; morphometry; elastin; lung structure

IT CAN BE ARGUED that the fundamental structure of the gas-exchanging lung parenchyma is the elastin fiber. Although illustrators have traditionally depicted alveoli as spheres attached to the airways as grapes are attached to their stem, the pulmonary parenchyma can be described more accurately as a collection of tents. The structure of a tent is a function of the geometry of the poles and ropes that support the canvas rather than of the characteristics of the canvas itself (Fig. 1).

Similarly, the structure of the lung parenchyma is determined by the supporting elastin fibers across which the epithelium is draped rather than by the epithelial cells themselves. It follows that the generation of new alveoli is likely a function of the development or alteration of parenchymal elastin cables rather than of the proliferation of epithelial cells.

The complexity of the pulmonary parenchyma increases postnatally out of proportion to the increase in lung volume in most mammals. This process is usually conceptualized as an increase in the number of alveoli. In many species, the process is most intense shortly after birth during a brief period of "septation" when large primitive air spaces are subdivided by new alveolar septa (4). This phenomenon involves the appearance of elastin cables within alveolar walls. These cables subsequently "lift up" or "bud" from the plane of the original or primary septum, creating a new alveolar septum. This process has been the subject of considerable investigation (4, 14–16, 23, 28, 30). Alveoli continue to appear postnatally beyond the period of septation, and there is intriguing evidence that parenchymal lung structure is remarkably plastic even in adult animals and that the potential for alveolar development may extend well into maturity (17, 26).

Little is presently understood about the mechanisms of alveolar generation beyond the period of septation (16). In some species, alveoli may continue to appear on airways that were previously without alveoli, thereby converting conducting airways into respiratory bronchioles (2). However, the number of airways that could be involved in this process is limited; so-called "centripetal alveolarization" could account for only a small fraction of the alveoli generated during this period. Three-dimensional reconstructions of alveolar ducts suggest that secondary septa could be added by septation to distal alveolar ducts (7). Although septation could continue to occur beyond the "critical period" (1), it might occur at an intensity that would be difficult to identify. It is also at least theoretically possible that alveoli could be added by the branching of alveolar ducts and the subsequent addition of new respiratory airway units. Clarifying the mechanisms of growth during this period of development is of particular interest given the recent realization that growth-regulating substances such as retinoids might be used to generate alveoli in conditions in which the alveolar number is abnormally low, such as chronic obstructive pulmonary disease or bronchopulmonary dysplasia (17).

One of the difficulties in understanding postnatal alveolar development has been the availability of few morphometric methods with which to address this issue: measurement of gas-exchanging surface area, characterization of alveolar size or number, and three-
dimensional reconstruction of whole acinar units. Unbiased techniques of alveolar morphometry have dramatically improved our understanding of parenchymal structure and alveolar development, but the parameters they provide (alveolar size, number, and surface area) are not easily translated into an appreciation of the mechanisms by which alveoli are generated. Three-dimensional reconstructions have been useful for characterizing the branching pattern of respiratory airways and alveolar ducts and the distribution and geometry of alveolar spaces within individual acini (7, 18). This labor-intensive technique is not, however, well suited to quantitative analysis of changes that might be unevenly distributed within or among acini.

We have used a novel morphometric technique that addresses the connective tissue structure of the lung parenchyma in immature and mature ferrets to understand better the mechanisms of postnatal alveolar development. This technique, developed by Oldmixon and colleagues (21, 22) to describe parenchymal architecture in adult dogs, is based on recognition of the various types of borders (or perimeters) of individual alveolar walls or faces. Slightly more than one-half of the lengths of alveolar septal borders in the dog are defined by elastin cables (Fig. 2, A and B). The majority of these cables either 1) occur in the free ends of alveolar septa and, on two-dimensional sections, define the “mouths” of alveoli or 2) are embedded within alveolar tissue and appear in two dimensions as an angular “bend” where two flat alveolar walls meet. Smaller numbers of alveolar walls end at parenchymal structures such as airways or vessels. In all of these cases, the elastin cable or parenchymal structure provides support for the alveolar wall. Slightly less than one-half of the lengths of alveolar walls comprise junctions where an alveolar septum meets two other alveolar walls (Fig. 2C). The three septa of a “junction” meet at nearly equal angles (20). The retractive forces

Fig. 1. Circus tent lung parenchyma analogy. Alveolus can be compared with a circus tent (A). Various walls of the tent are flat as are walls or faces of alveolus. Ropes support canvas of the tent in the same way that elastin fibers of parenchyma support gas-exchanging walls of alveoli. When the 2 components of the circus tent are separated, it is clear that geometry and structure of the tent is determined by its supporting poles and ropes (B) rather than by canvas shell (C). Likewise, changes in parenchymal anatomy with growth are likely determined by alterations in connective tissue skeleton.

Fig. 2. Three-dimensional structures of the 3 major alveolar border types identified on 2-dimensional section as ends, bends, and junctions (A–C, respectively). D: schematic of theoretical process of septation and its effect on alveolar border lengths. In each schematic [adapted from Oldmixon et al. (21)], a load-bearing elastin fiber under longitudinal tension is represented as an exaggerated cable. Arrows represent forces on the border resulting from surface tension and elastic properties of alveolar walls. A: ends are free borders supported by an elastin cable. These borders represent mouths of alveoli along alveolar duct. B: a bend consists of an elastin cable under tension within an alveolar wall that creates a pup tent-like surface. This border is recognized in 2 dimensions as an angular meeting of 2 flat alveolar walls with an elastin cable at the angle. C: junctions comprise confluence of 3 adjacent alveoli in which the 3 alveolar walls join at approximately equal angles. At this border, retractive forces of alveolar walls and surface tension balance, and there is no elastin cable necessary or present. In 2 dimensions, this border type represents a 3-way intersection with nearly equal angles. D: when the angle (ß) of a bend (B) changes with growth, elastin cable might lift off from the joining of the 2 alveolar walls and thus create a new alveolar wall. This process (septation) should decrease length of bends and increase length of junctions (J) and ends (E), the latter 2 being created by this process.
of the three alveolar walls is balanced, and no net force is produced. This structure is therefore stable despite the absence of an elastin cable (20). In adult dogs, the lengths and distribution of the various types of alveolar septal borders can be identified by light-microscopic analysis in sections stained for elastin, and the distribution of these categories is relatively constant among individuals (21).

We hypothesized that the distribution of the various alveolar septal border types should be altered in a predictable way if septation was the predominant process of later postnatal alveolar development. If parenchymal development in this period involves septation, elastin cables that are originally within alveolar tissue (bends) might be converted to ends, as illustrated in Fig. 2D. This process would also result in the creation of a new junction. Therefore, we reasoned that measuring changes with age in the total lengths of the various alveolar borders and their proportional distributions might help elucidate the structural processes of postnatal alveolar generation and complement information that has been gained by other techniques.

METHODS

We studied the lungs of weanling (8- to 10-wk) and adult (>2-yr-old) male ferrets obtained from Marshall Research Animals (North Rose, NY). The protocol was approved by the University Committee on Animals Research. The animals were anesthetized (60 mg/kg of intraperitoneal pentobarbital sodium and 25 mg/kg of intramuscular ketamine), and tracheostomies were performed. The animals were ventilated, the heart and lungs were exposed through a median sternotomy, and heparin sodium (1,000 units/kg) was injected into the right ventricle. The pulmonary artery and left atrium were separately cannulated, and the lungs were perfused with oxygenated, warmed (38°C), buffered (pH 7.4) lactated Ringer solution containing 2,000 µg/l of sodium nitroprusside. Transpulmonary pressure measured at the trachea was cycled between 3 and 30 cmH2O with 95% O2-5% CO2 as the Ringer solution. During perfusion, the lungs were excised and suspended from the tracheal cannula. Three pressure-volume loops were generated to establish and confirm a consistent pressure-volume relationship. The lungs were then deflated from 30 to 15 cmH2O and held there during fixation and dehydration. From this point, perfusion pressure was maintained at 10–20 cmH2O above airway pressure by adjusting the rate of a peristaltic pump. The lungs were fixed by vascular perfusion with 200–400 ml of a mixture of 2% glutaraldehyde, 1% formaldehyde, and 1% tannic acid or with 2.5% gluteraldehyde followed by 2% tannic acid (pH = 6.9). After fixation, the fixativesolution was flushed from the vasculature by perfusion with a small volume of lactated Ringer solution. The lungs were then perfusion dehydrated (22) with 400–800 ml of an ethanol gradient, increasing from 10 to 100%, while the transpulmonary pressure was maintained. Each lung was then sliced into 10 equal coronal slices beginning at a random point in the first interval, and the volume of fine parenchyma in the fixed dehydrated lungs was determined by point counting. Random blocks, ~4 × 7 mm, were embedded in glycol methacrylate (B-4, Polysciences) and 2-µm sections were cut and mounted on slides for light microscopy. Changes in dimensions during processing were assessed by comparing the dimensions of the preembedded block face with those of the tissue sections on slides. Sections were stained for elastin (6) with Miller’s stain (Polyscientific) and lightly counterstained with 1.5% phloxine B (J. T. Baker).

Randomly selected fields were photographed with high-contrast black-and-white film (Kodak Tmax100) and a Wratten no. 22 orange filter. Negative images were projected for morphometric analysis at a final magnification of ×1,000. Two readers (Wood and McBride), blinded to animal type, point counted the fine parenchymal area on each field. Alveolar septal borders were identified and counted. Septal borders were categorized as ends, bends, junctions, structure borders, respiratory bronchiole borders, or either of two less-common types, “X” or “T” (21). Border traces that displayed a “free end” that stained for elastin were considered ends. Ends occurred where two septal traces, themselves straight, met at an angle, typically 130–150°, with elastin staining present at the angle. The confluence of three septal traces with no angle > 180° comprised a junction. Elastin staining was absent from junctions. A structure border occurred when a septal trace terminated at the wall of a larger structure such as a blood vessel or conducting airway. A respiratory bronchiole border occurred when a septal trace terminated at an alveolated airway with a knob of tissue that included airway epithelium. Both structure borders and respiratory bronchiole borders contained large amounts of elastin staining, occasionally in a striated pattern. Four septal traces meeting with angles < 180° constituted an X border. A T border represented the junction of three septa in which one of the angles was 180° or greater and considerable amounts of tissue and elastin staining were present. These last two morphologies (X and T) were infrequent. They are not unique septal border types themselves but have been shown to represent sections that pass through several bends and/or junctions simultaneously (21). Sections were considered acceptable for analysis when all ends and none of the junctions demonstrated elastin staining (see Fig. 3). The two observers reviewed any fields with >30% disagreement in end, bend, or junction counts. These fields were independantly recounted.

Length densities of the various septal borders were calculated by multiplying the number of profiles identified by two and dividing by the summed areas of the fields examined (9). Absolute border lengths of each septal border type in each lung was then calculated by multiplying the appropriate length density by the volume of fine parenchyma in each fixed dehydrated lung. To characterize the extent to which alveolar border-type lengths increased in proportion to the increase in lung volume with growth, absolute border lengths were normalized by the cube root of fine parenchymal volume.

Statistics. We modeled border lengths as a function of adult or weanling status. Border counts and corresponding calculated lengths were more variable in adult animals than in weanlings. We modeled these differences to assume a variance for the process that is proportional to the mean value. Furthermore, we accounted for the statistical dependence among observations that resulted from analyzing multiple slides from each animal.

We employed the method of generalized estimating equations (8, 13) to determine point estimates ± SE for estimates of a multiplicative effect of adult-to-weanling status on the lengths of each of the alveolar septal borders. This method also allowed us to add a term in our model representing the log of lung volume to confirm that the proper power of lung volume was used to scale the lengths.

To compare the difference between the lengths of weanling and adult alveolar septal borders, this model was also used to fit the collection of all border counts, with terms representing the mean level of each of the borders and the proportions by
which each of them changed. This allowed comparison between the amount by which lengths varied from weanlings to adults.

RESULTS

Weanling animals (n = 4) weighed 0.34 ± 0.02 (SE) kg and mature animals (n = 5) weighed 1.80 ± 0.14 kg. Volume of the fine parenchyma was 33.2 ± 2.5 and 129.8 ± 4.8 ml for weanlings and adults, respectively. Section area was greater than preembedded block face area by a similar degree in each age group (13.0 ± 1.9% in weanlings and 14.8 ± 1.7% in adults). Correcting for this difference did not influence the findings. Forty fields from weanling lungs were counted by both readers, yielding a total of 6,315 borders. Thirty-eight adult fields yielded 2,928 borders.

Mean absolute lengths for each of the border types in each group and lengths normalized for lung volume are shown in Table 1. Absolute lengths were similar among animals in each group and, as expected, were all much greater in mature animals. However, the lengths of ends and bends, when normalized for fine parenchymal volumes, were longer in mature animals by 23 and 37%, respectively. The 17% difference in normalized junction lengths was not significantly different (P = 0.10). Normalized bends increased out of proportion to both ends and junctions and ends increased out of proportion to junctions (P < 0.0001 for all comparisons). T borders decreased by 38% but represented only 2% of the total counts.

The relative distributions of border types at both ages are presented and compared in Table 2. In both groups, ends, bends, and junctions comprised >90% of the total alveolar septal border length. In both weanlings and adults, ends represented 22% of the total alveolar septal length. Although bends represented a greater percentage in adults than in weanlings (23.8 vs. 21.4%), junctions represented a smaller percentage (44.4 vs. 46.3%). T-border length decreased from 1.5 to 0.8%.

DISCUSSION

The anatomy of the pulmonary parenchyma reflects the geometry of 1) the elastin cables that define the mouths of alveoli along alveolar ducts and 2) the continuations of those cables that lie within the alveolar walls. Evaluation of this network is difficult given that the majority of lung elastin is in vessels and airways. Elastin content in the lung apparently increases very little with growth beyond the time of seption (24) but can be induced by a variety of insults (3). Once generated, elastin is remarkably permanent in the lung (27). Although a number of investigators

Table 1. Absolute and normalized lengths of alveolar septal borders

<table>
<thead>
<tr>
<th>Border</th>
<th>Absolute Length, mm × 10^9</th>
<th>Length/Volume^1/3, × 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>5.68 ± 0.26</td>
<td>1.13 ± 0.36</td>
</tr>
<tr>
<td>B</td>
<td>5.47 ± 0.49</td>
<td>1.13 ± 0.42</td>
</tr>
<tr>
<td>J</td>
<td>11.81 ± 1.14</td>
<td>21.90 ± 1.33</td>
</tr>
<tr>
<td>F</td>
<td>1.34 ± 0.30</td>
<td>3.00 ± 0.45</td>
</tr>
<tr>
<td>R</td>
<td>0.59 ± 0.09</td>
<td>1.13 ± 0.13</td>
</tr>
<tr>
<td>X</td>
<td>0.08 ± 0.02</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>T</td>
<td>0.38 ± 0.06</td>
<td>0.40 ± 0.07</td>
</tr>
</tbody>
</table>

Values are expressed as length densities of alveolar septal borders.

Table 2. Length densities of septal borders

<table>
<thead>
<tr>
<th>Border</th>
<th>Length Density, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weanling</td>
</tr>
<tr>
<td>E</td>
<td>0.225</td>
</tr>
<tr>
<td>B</td>
<td>0.214</td>
</tr>
<tr>
<td>F</td>
<td>0.463</td>
</tr>
<tr>
<td>R</td>
<td>0.057</td>
</tr>
<tr>
<td>X</td>
<td>0.023</td>
</tr>
<tr>
<td>T</td>
<td>0.015</td>
</tr>
<tr>
<td>Total</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Values are expressed as proportion of total length density. A/W, ratio of adult to weanling length density; CI, confidence interval. Data on adult dogs are from Oldmixon et al. (21). Theoretical data are as predicted by Fung (10). *P < 0.0001 for A/W values ≠ 1.00.
have specifically identified changes in parenchymal elastin fibers with growth (3) and after pneumonectomy (12), how these changes affect the development of gas-exchange surfaces is not clear.

Oldmixon et al. (21) published a series of studies that elegantly describe the anatomy of parenchymal elastin fibers in the dog. One contribution of these studies is an appreciation of the elastin fibers that lie within the alveolar walls rather than in the alveolar entrance rings. These fibers are under tension and are referred to as bends. The authors used three-dimensional reconstruction to show that end elastin fibers often leave the mouths of alveoli and follow a path within alveolar septa where they appear as bends on two-dimensional section. Remaining within a single alveolar duct and its associated air spaces, they most often return to the mouths of alveoli where they again become ends defining the alveolar duct. These cables presumably carry a portion of the forces that would be born entirely by alveolar walls themselves if the axial connective tissue network of the airways ended with the alveolar entrance rings. Bends also distort the alveolar wall and increase the surface-to-volume ratio of the air spaces. In dogs, the lengths of bend cables are nearly the same as those of ends. It is possible that lung growth involves the conversion of bends to ends with the creation of new alveolar walls as illustrated in Fig. 2D. Butler et al. (5) pointed out that if the tension on a bend changes such that the angle between the two alveolar walls becomes <120°, separation of the cable from the junction of the two walls, with creation of a new alveolar septum, would minimize local surface area and energy. If this were the case, the conversion of bends to junctions and ends (septation) could increase the number of alveoli and the alveolar surface area by creating new secondary septa in preexisting alveolar ducts. It is attractive to consider that this minimization of local energy could be one of the factors that determines the alteration in parenchymal anatomy with growth. In this regard, bend cables created early in life could represent a “reservoir” of potential alveolar septa that could be formed later in life without the addition of new elastin cables.

We found that the lengths of the various types of alveolar borders increased with lung growth between 8 and 10 wk and maturity (2 yr of age) in the ferret. Over this time period, body weight and lung volume each increased approximately three- to fourfold. The lengths of the two major types of alveolar borders containing elastin cables, ends and bends increased out of proportion to lung volume. This increase in normalized alveolar border lengths reflects the fact that the lung parenchyma did not merely expand but became more complex, a phenomenon that could theoretically involve an increase in the number of alveoli and/or an increase in the geometric complexity of individual alveoli.

The distribution of alveolar border lengths we found in the ferret was remarkably similar to that found by Oldmixon et al. (21) in the adult dog. The proportions of each type of alveolar border in the weanling ferret, adult ferret, and adult dog are illustrated in Table 2.

The close agreement of these proportions and the small confidence intervals for these categories in both studies suggest not only that parenchymal structure is remarkably similar in these two different species but also that the technique itself is robust.

The successful application of this technique depends on attention to a number of technical issues. Plastic embedding is required for preparing adequate sections for analysis. Fresh commercial Miller’s stain was adequate for tissue embedded in the glycol methacrylate material we used. It is necessary to preserve the lung at a relatively high volume so that the alveolar walls are linear and bends can be reliably identified (19). Oldmixon et al. (22) argued that perfusion fixation and dehydration optimally preserve parenchymal architecture, but it is possible that this technique could also be used with tissue fixed at a high volume via the airways. With optimal preservation of parenchymal architecture, recognition of various border types was relatively straightforward. With practice, there was a high degree of agreement between the two observers. Although there was little difficulty applying this technique to both weanlings and adults, we were unable to extend this study to the analysis of very immature lungs because of the thickness of the alveolar walls.

The implications of the distribution of alveolar border types for the structure of the lung parenchyma is not straightforward. The measured distribution of border types in ferrets and dogs differs dramatically from predictions of this distribution from at least one theoretical model of parenchymal structure. Fung (10) has proposed a model of the lung parenchyma that satisfies the constraints that the alveoli be connected to a branching tree and that the alveoli and airways completely fill the volume of the lung. This model predicts that the distribution of alveolar border lengths would be 1.4% ends, 9.6% bends, and 89% junctions. The marked discrepancy between these predictions and independent measurements in the ferret and dog (Table 2) suggests that the pulmonary parenchyma does not closely confor to this model in either of these species.

It is also difficult to arrive at firm conclusions about the mechanisms of postseptation parenchymal development from the measurements of alveolar border lengths. Nevertheless, these data have a number of potential implications. If the additional complexity of the lung parenchyma were a result of the addition of new units structurally similar to preexisting units, then the distribution of border lengths might be expected to remain the same. Alternatively, septation would be expected to result in an relative increase in the lengths of ends and junctions regardless of whether it involved the conversion of preexisting bends to ends and junctions (as illustrated in Fig. 2D) or the creation of new elastin cables. In planning this study, we expected to find such a change in border length distribution (increased ends and junctions and decreased bends). We were surprised to find a greater increase in bends than in either of the other two border types.

The greater increase in bends with postnatal growth raises the possibility that one of the processes involved
in postseptation parenchymal development is an increase in the complexity of individual alveoli, such as would be created by adding an elastin cable within an initially flat alveolar wall, applying tension to the cable, and creating a crease in the wall to increase its surface area. Bends are not a major component of the model of parenchymal architecture proposed by Fung (10) or of intuitive notions of parenchymal architecture based on the concept that the acinus resembles a cluster of grapes. For instance, the classic model of the alveolar duct posited by Wilson and Bachofen (29) focuses on the mechanical properties of elastin fibers that comprise the mouths of alveoli and correspond to ends on two-dimensional sections. In both the dog and the ferret, the length of elastin fibers in bends is approximately equal to or greater than that in ends. Our finding that the length of bends increases out of proportion to that of ends and junctions suggests that some process in which elastin fibers develop within alveolar walls is responsible for at least a portion of the increase in gas-exchanging surface area postnatally.

Alternations in parenchymal geometry unrelated to the generation of new elastin cables could influence the distribution of alveolar border lengths. For instance, a change in the relative proportion of volume within the alveolar duct compared with that within the alveoli could have important effects. A decrease in the relative volume of alveolar duct air as opposed to alveolar air should decrease the relative lengths of ends and increase the lengths of bends and junctions. Measuring changes in these relative volumes with certainty is difficult on two-dimensional sections (11, 25), and we did not make this measurement. However, this change alone is unlikely to explain our data because the length of junctions did not increase in proportion to the increase in bends.

In conclusion, our findings confirm that alveolar complexity increases with growth and suggest that the appearance of elastic fibers in alveolar walls may play a pivotal role in this increased complexity. Furthermore, our findings do not support the hypothesis that septation is the primary mechanism of growth during this period.

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