Differences in airway structure in immature and mature rabbits

R. RAMCHANDANI,1 X. SHEN,1 C. L. ELMSLEY,3 W. T. AMBROSIIUS,3 S. J. GUNST,2 AND R. S. TEPPER1

1Department of Pediatrics, 2Department of Physiology and Biophysics and 3Division of Biostatistics, Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana 46202

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PREVIOUS STUDIES HAVE SHOWN that bronchoconstriction with methacholine produces a greater increase in pulmonary resistance in immature than in mature rabbits (33, 34). This maturational difference in the effect of methacholine on lung resistance is primarily caused by greater airway narrowing in the immature rabbits (31). The mechanisms for the maturational differences in airway narrowing remain unclear. The degree of airway narrowing depends on the smooth muscle shortening, the structure of the airway wall, and its interaction with the lung parenchyma. The magnitude of smooth muscle shortening is determined by a balance of two forces: the force generated by the airway smooth muscle (ASM) and the forces that limit ASM shortening (13, 15, 22, 43). A greater amount of ASM within the airway wall could increase the force generation and thus increase airway narrowing in immature rabbits. Conversely, less cartilage in the airway wall could result in a more compliant airway and greater airway narrowing. In addition, fewer alveolar attachments to the immature airway wall could decrease the forces of interdependence between the airways and the lung parenchyma and could thus decrease the load that ASM must shorten against. Furthermore, geometric factors, such as the thickness of the airway wall internal to the ASM relative to the airway diameter, can also affect the degree of airway narrowing. For the same degree of ASM shortening, a proportionately thicker airway wall internal to the ASM will amplify the effect of any smooth muscle shortening and thus lead to greater airway narrowing (22).

Our laboratory has previously determined that there is greater airway narrowing in the immature than the mature rabbit lung (33). The purpose of the present study was to determine whether there were structural differences in the airways of immature and mature animals that may account for these functional differences. In this study, we compared the following airway morphometric characteristics, which might contribute to greater airway narrowing in immature rabbits: 1) the fraction of airway wall occupied by ASM; 2) the fraction of airway wall occupied by cartilage; 3) the ratio of wall area internal to the ASM relative to the area of the airway; and 4) the number of alveolar attachments to the airways.

METHODS

A total of 11 immature (4–6 wk, 0.5–0.6 kg) and 10 mature (6 mo, 2.5–2.7 kg) New Zealand White rabbits were studied. Animals were anesthetized with pentobarbital sodium (50 mg/kg body wt) and then exsanguinated by severing of the abdominal artery. The intact lungs were then excised and degassed under vacuum. A canula was tied to the proximal portion of the trachea, and the lungs were inflated with 10% formalin to a distending pressure of 20 cmH2O. The lungs were fixed at 20 cmH2O for 4 days using a recirculating pump, after which the lungs were immersed in 10% formalin for an additional 3 days to ensure complete fixation of the tissue. Two preparations were used: 1) isolated airway preparation and 2) whole lung preparation. The distending pressure of 20 cmH2O was chosen so that the airways would be
maximal in size (to minimize mucosal folding) and would be nearly circular, which would minimize identifying airways cut longitudinally vs. buckled airways at lower transpulmonary pressures. In addition, our previous data indicate that maximal diameter is actually achieved above 10 cm H₂O.

**Isolated Airway Preparation**

To compare the structural characteristics of the airways from immature and mature rabbits, we isolated the airways of comparable anatomic location from six immature and five mature rabbits. The lung parenchyma was scraped from the main axial pathway of the right lung, and the isolated airway was divided into eight segments, each representing a generation. Each segment was the portion of the axial pathway between two successive major branches arising from the primary axial pathway. The trachea was numbered generation 1, and the most distal isolated portion of the right axial pathway was generation 8 (Fig. 1). Each airway segment was individually embedded in paraffin, and 5-μm-thick sections were cut from each paraffin block and stained using Masson's Trichrome technique. Sections were cut from the distal portion of each segment to minimize the variability associated with sampling across generations and rabbits. Airway sections in which the ratio of the largest to smallest diameter was >2 were excluded to minimize using airways that had not been sectioned perpendicular to the longitudinal axis. We excluded six immature and four mature airways that did not fit this criterion. In addition, the ratio of larger to smaller diameters for airways included for analysis did not differ for the immature and mature animals. Airway sections were visualized by light microscopy, and images were captured using a digital camera (DC-120, Kodak, Rochester, NY) coupled to the microscope. Morphometric analysis of the images was performed using an imaging software program (Metamorph, version 2.76, Universal Imaging), with computer-assisted tracing of the internal perimeter and airway wall thickness.

**Morphometric Measurements**

**Airway dimensions.** The following measurements of airway dimensions were obtained from sections of each airway generation: 1) internal perimeter of the airway defined by the

\[ P_i = \pi R_{mo} \]

\[ P_i = P_i / 2\pi \]

\[ T_i = T_i \]

\[ T_i = \pi R_{mo}^2 \]

\[ R_{mo} = r_i + T_i \]

The absolute inner wall area (WAₐ), was calculated as the difference of Aₐ and Aₜ:

\[ WA_i = A_{mo} - A_i \]

The proportional inner wall area (PWA_i) was calculated by taking the ratio of the WA_i to the sum of WA_i and A_i and expressed as a percentage [PWA_i = WA_i / (WA_i + A_i) × 100].

A single observer, who was blinded to the identity of the photomicrographs, performed the morphometric analysis of the airways. The average percent coefficient of variation for measurements of thickness was 2.7% and for perimeter was 1%.

**Airway wall composition.** For each of the isolated airways taken from the six immature and five mature rabbits, the fractions of the airway wall occupied by ASM and cartilage

![Fig. 1. Isolated airways from a mature rabbit. Numbers indicate generation, with the trachea labeled as generation 1.](image-url)
were determined by point counting (41). A $10 \times 10$ grid was superimposed on the downloaded image of the airway on the computer screen using the Metamorph software. Each of the digitized images was taken from the four quadrants for each airway. The value reported for each airway was determined by averaging measurements from the four quadrants. The percentage of the wall components for each airway was estimated by counting the number of points of intersection for each of the components and then expressed as a fraction of the total number of components (total airway wall).

In a subset of the sample, point counting was done in triplicate to determine the reproducibility of the measurements. The average percent coefficient of variation for the measurements of percent muscle was 5.0% and for percent cartilage was 3.6%.

**Whole Lung Preparation**

Alveolar attachments and airway dimensions. To evaluate the number of attachments to the intraparenchymal airways, the formalin-fixed right lungs from five immature and five mature rabbits were cut into five, equally spaced, transverse sections (referred to as whole lung preparation). Each piece was then embedded in paraffin, sectioned, stained, and visualized under a light microscope. Digital images were captured as described in *Isolated Airway Preparation*. Airways with a ratio of largest to smallest diameters $>2$ were excluded. Each airway image was visually inspected, and the number of alveolar attachments was counted around the outer perimeter using the Metamorph software. $P_i$ was measured as previously described for the isolated airway preparation.

**Statistical Analysis**

Repeated-measures ANOVA was used to evaluate maturational differences in the outcome variables across all generations of isolated airways (16). Included in these models were linear effects of generation. For muscle and cartilage, we also included a quadratic effect for generation, as was indicated graphically. An overall $P$ value for differences between mature and immature rabbits was calculated for each of the response variables ($D_i$, $T_i$, PWA$_i$, percent cartilage, and percent muscle). The relationship between the number of alveolar attachments and $P_i$ was modeled using a repeated-measures ANOVA model, with the logarithm of the number of attachments as the response and the logarithm of $P_i$ and maturity as predictor variables. We tested for a difference in slopes between mature and immature rabbits by including the maturity by perimeter interaction.

**RESULTS**

Mean $D_i$ and $T_i$ for immature and mature rabbits are plotted as functions of generation in Fig. 3, A and B. $D_i$ and $T_i$ decreased with increasing generation for both immature and mature airways. Airways from the mature rabbits had larger absolute $D_i$ ($P < 0.001$) and greater $T_i$ ($P < 0.002$) compared with those from immature rabbits. PWA$_i$ increased with increasing generation for both age groups; however, the difference in PWA$_i$ in airways from the immature and mature rabbits across generations was not statistically significant ($P = 0.24$; Fig. 4). As an exploratory analysis, we examined the wall areas seen in Fig. 4 in greater detail. There was no evidence that a difference existed at generations 2, 3, 4, 6, 7, and 8 ($P > 0.1$ for all). At generation 5, there was slight evidence that mature rabbits tended to have larger wall areas ($P = 0.0149$, unadjusted for multiple testing). A likely reason for not observing differences at other generations was lack of power due to small sample sizes, with estimated power ranging between 5 and 31%.

The percentage of the airway wall occupied by smooth muscle increased from the central to the more peripheral airways for both groups (Fig. 5). The mean percentage of ASM across generations was greater in the immature than in the mature airways, and the difference approached statistical significance ($P = 0.06$). In contrast to the increase in ASM with generation, the percentage of cartilage in the airway wall decreased as generation number increased, for both groups (Fig. 6). In addition, the immature rabbits had a smaller mean percentage of cartilage across generations compared with mature rabbits ($P = 0.03$).

A total of 56 immature and 152 mature airways were analyzed from the whole lung sections. The number of
alveolar attachments increased with increasing $P_i$, as seen for the log-transformed data for both mature and immature airways ($P < 0.0001$; Fig. 7). For the same airway size, the immature rabbits tended to have fewer attachments ($P = 0.07$), but there was no evidence that the relationship between the number of alveolar attachments and $P_i$ differed between mature and immature rabbits ($P = 0.59$).

**DISCUSSION**

The results of the present study demonstrate that there are structural differences in the anatomically similar airways of mature and immature rabbits that may contribute to the greater airway narrowing previously observed in these animals (33). The airway walls of immature rabbits have proportionately more smooth muscle, which could result in greater force generation and shortening. A lower percentage of cartilage was also observed in the airway wall of immature rabbits compared with mature rabbits. This difference could also contribute to greater airway narrowing in the immature animal by providing a lower load during shortening of the smooth muscle. The immature rabbit airways also had fewer alveolar attachments compared with mature rabbit airways, which could result in decreased interdependence between the airways and the lung parenchyma in immature animals, and thus a smaller load to resist ASM shortening. We did not find that immature airways have greater airway $T_i$ relative to airway size for comparable airway generations. This suggests that geometric factors, such as a relatively thicker wall, may not contribute to the greater airway narrowing in immature rabbits.

**ASM**

The proportionately greater ASM in the airway wall of the immature than the mature rabbits may be a basis for our laboratory’s previous findings of greater airway narrowing in immature animals. Computational models of airway narrowing have demonstrated that the degree of airway narrowing is linearly related...
to the proportion of smooth muscle in the airway wall (22). Our findings of a relationship between the proportion of smooth muscle in the airway wall and the amount of airway narrowing in immature and mature rabbits is consistent with studies assessing the relationship between the proportion of ASM and airway narrowing in mature animals of different species. Martin and co-workers (18) reported that a higher proportion of ASM within the airway wall correlated with greater maximal increases in airway narrowing across different mammalian species.

There are few studies that have evaluated maturational differences in the quantity of ASM in the airway wall. Matsuba and Thurlbeck (19) compared the quantity of ASM in the airways of human children and adults. These investigators found that the proportion of ASM in large airways was similar in the two groups, but that the children had a smaller proportion of ASM in the smaller airways. Although the reasons for the differences in overall trend in our study and that of the human study could be many, one of them could be attributed to differences in the method of classification of the studied airways. In our study of rabbits, we evaluated anatomic comparably comparable airways. In contrast, the human airways were grouped as major bronchi or airways <2 mm in diameter; thus the airways were not anatomically matched. In a study of swine (23), the percent of ASM in the airway wall was compared in 2- to 10-wk-old animals through generations 2 to 4 and was found to be similar; however, airway rings from the 2-wk-old animals produced greater maximal tension. In swine, there were no differences in percent smooth muscle in generations 0–5 in 2- and 10-wk-old animals (23). We found that the proportion of ASM within the airway wall increased within the more distal generations in both age groups, a finding that is similar to observations in humans (19, 32, 39). Therefore, the swine airway may be structurally different than those of humans and rabbits and thus may have a different relationship between structure and function with respect to airway reactivity.

**Cartilage**

In our study, there was a smaller proportion of cartilage in the airway wall of the immature than the mature rabbits. This finding is consistent with the observations of maturational differences in the structure of airways in sheep (6, 29). In humans, one study reported a lower fraction of cartilage in the airway walls of infants than adults (11); however, another study found no maturational differences in cartilage in the airway wall (19). A lower proportion of cartilage could result in a more compliant airway wall. Studies of airways from rabbits, humans, and several other species have found that immature animals have more compliant airways (4–6, 20, 27, 29).

Cartilage provides structural support to the airway wall and is an elastic load that resists airway narrowing. In rabbits, softening of the cartilage tissue by administration of intravenous papain increases the maximal airway response to acetylcholine (21). This suggests that increasing the compliance of the cartilage decreases the load that the ASM shorts against and results in greater airway narrowing. Cartilage can provide a preload that determines ASM length, as well as an afterload during ASM shortening. When tracheal smooth muscle shortens, the horseshoe-shaped cartilage is deformed away from its resting position, creating an elastic load that opposes ASM shortening (9). In intrapulmonary bronchi, overlapping of the cartilage plates can provide a significant load to ASM shortening (26, 39). Maturational differences in the quantity of cartilage within the airway wall may affect airway narrowing by altering the elastic pre- and afterload, such as when the cartilaginous plates overlap and limit airway narrowing.

**$T_i$ and $A_i$**

$PWA_i$ increased in the more distal airways in both immature and mature rabbits; however, we did not find that immature animals had proportionately thicker airway walls internal to the ASM. Our observation that the proportional inner wall increases with increasing generation is similar to results reported for other species (12, 24, 25). $T_i$ can greatly amplify the degree of airway narrowing and may be a mechanism for heightened airway reactivity in patients with asthma and chronic obstructive pulmonary disease (28, 42). However, in our study, the absence of differences in $A_i$ relative to airway size between mature and immature rabbits and a tendency for the mature to have greater values suggests that wall thickness does not account for the greater airway narrowing in immature than mature rabbits. However, in a previous study, our laboratory found that immature rabbits had proportionately thicker airway walls (31) after bronchoconstriction. It is therefore possible that other factors, such as airway wall edema produced during bronchoconstriction, may result in greater increases in $T_i$ and thus greater airway narrowing in the immature animal. This mechanism would be consistent with the report of greater airway wall leak in immature than mature guinea pigs after intravenous histamine (2).

**Number of Alveolar Attachments**

The immature rabbit airways had fewer alveolar attachments than mature rabbit airways of similar size. In addition, the number of attachments increased with increasing airway size for both age groups. This latter finding is consistent with a study in dogs (25), as well as a study in humans (30). We are not aware of any previous reports regarding changes in the number of alveolar attachments with lung growth and maturation. However, as the lung undergoes alveolarization early in life, one would expect that the number of attachments to the airways might increase with lung growth. In fact, rats and mice have mostly sacculles and not alveoli at birth and fairly rapid alveolarization very early in the postnatal period (1, 38). Rabbits and humans are born with a small number of alveoli that
increase in size and number during lung growth (36, 37). Physiological studies and computational models of airway narrowing have highlighted the importance of the forces of interdependence between the airways and the surrounding lung parenchyma in limiting airway narrowing (7, 10, 14, 40). In addition to the elastic load from the pulmonary recoil pressure of the lung parenchyma, there is an additional elastic load caused by local distortion of the lung parenchyma surrounding the airway as the ASM shortens and the airway narrows (3). This local distortion can be quantified in terms of a shear modulus. In a recent study in rabbits, our laboratory found a lower shear modulus in immature than mature rabbit lungs. In contrast, the number of alveolar attachments in the immature animal is similar to the ~15% fewer alveolar attachments for immature compared with mature rabbit lungs.

Structure-Function Relationships

We attempted to evaluate the effects of our observed maturational differences in airway structure on airway narrowing by using simple computational models that have been proposed in the literature (17, 22). The effect of an increased proportion of ASM was assessed using the model proposed by Moreno et al. (22) in 1986. The predicted maximal fold increase in resistance during bronchoconstriction was 1.5-fold greater for the immature compared with mature rabbit lungs. We conclude that these structural differences in the airway wall may contribute to the greater maximal airway narrowing observed in immature rabbits during bronchoconstriction.

REFERENCES