Postnatal alveolar development of the rabbit

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Received 16 October 2001; accepted in final form 17 April 2002

Although studies of lung growth and development in humans are limited by the availability of normal lungs, ~10% of alveoli are thought to be present at birth (3). However, the end point of alveolar formation remains unclear, with estimates ranging from 6 mo to 8 yr of age (7, 10, 34, 35, 38). The small sample size and the apparently large variation in the total adult number of alveoli (200–600 million) (34) have limited definitive conclusions. Rats and sheep are commonly used as models for human postnatal alveolar development; however, the majority of rat alveoli are formed entirely postnatally in the first 2 wk of life (4, 5), and sheep already have most of their alveoli at birth (8). Thus, in terms of the timing of onset of alveolarization, the rat and sheep may not be the most suitable model for postnatal human alveolar development.

The rabbit has been extensively used in respiratory research as a model for studying the impact of antenatal steroids (12, 27, 28) and surfactant replacement (9, 11, 14–16, 19, 21) and for the comparison of lung mechanics between immature and mature lungs (17, 22–24, 29–33). The aim of this study was to comprehensively characterize the postnatal alveolar development of the rabbit and to determine whether it may be a suitable model for human postnatal alveolar development.

MATERIALS AND METHODS

Tissue preparation and sampling. This protocol was approved by the TVW Telethon Institute for Child Health Research animal experimentation committee. New Zealand White rabbits (n = 3–7 per group) between 28 days gestation (dg) and 9 mo of age were weighed and given a lethal overdose (5 ml/kg ip) of pentobarbital sodium (325 mg/ml). The age groups studied were 28 dg, birth, 1 day, 3 days, and 1, 2, 4, 6, 8, 12, 16, 24, and 36 wk. Lungs were excised, and inflation was fixed overnight with 4% paraformaldehyde (325 mg/ml). Fixed lung volume (FLV) was measured by volume displacement (20). The right lung was removed and divided into the anterior azygous and right anterior and right posterior lobes. A sample was systematically removed in the sagittal plane from the center of each lobe. All morphometric measurements were performed blind on 5-μm hematoxylin- and eosin-stained sections.

Volume fractions. Volume fractions of lung parenchyma (parenchymal fraction (PF)) (alveoli and alveolar ducts), non-parenchyma (conducting airways and blood vessels), and pleura were estimated from photographic enlargements ×23 of 5-μm sections by superimposing a cycloid point counting grid (74 lines/148 points). Volume fraction is defined as PF/PM, where PM is the number of test points in contact with the structure of interest (e.g., parenchyma) and PF is the total number of points hitting the reference space (total of all compartments). Parenchymal volume (PV) in the right lung was derived from fixed lung volume as follows

$$PV = PF \times FLV$$

Parenchymal morphometry. A Sony 3CCD color video camera interfaced with a Leica DMLS microscope and a Macintosh 8100/80AV computer were used to capture 10 random nonoverlapping digitized images from each 5-μm section. The final magnification of the images was ×220. A linear point counting grid (21 lines/42 points) was superimposed onto the images, and the number of points falling on alveoli, ducts, or septal tissue and the number of air-tissue tissue-air intercepts were counted. Alveolar (AF), duct (DF), and tissue

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fractions (TF) were calculated by using $P_t/P_i$. In transverse sections, alveoli were identified as those structures opening onto a common airspace (alveolar duct). The relative size and the presence of secondary alveolar septa distinguished alveolar ducts from alveoli. In cross sections, alveoli were identified as those structures opening onto a common airspace (alveolar duct). The relative size and the presence of secondary alveolar septa distinguished alveolar ducts from alveoli. In cross sections, alveoli were identified on the basis of size (∼50–150 μm; see Fig. 1) and shape (circular). Irregular or ambiguous structures (on average 8%) were rejected. The total number of alveoli ($N_T$) in the right lung was calculated by the following formula

$$N_T = N_V \times \frac{PV}{100}$$

where $N_V$ is the number of alveoli per unit volume. The method described by Weibel (37) was used to calculate the alveolar numerical density by the following formula

$$N_A = \frac{N_V^{3/2}(\beta \times AF^{3/2})}{D}$$

where $N_A$ is number of alveoli per unit area, $\beta$ is a shape constant describing alveolar shape (1.55), and $D$ is a distribution variable of the characteristic linear dimension of the alveoli (equal to 1). The average alveolar volume for each age group was calculated by

$$V_A = \frac{(PV/100 \times AF)}{N_T}$$

The average surface area of alveoli ($SA_A$) was determined by multiplying the surface fraction by lung volume and the P寂静物

$$SA_A = S_V \times FLV \times PF$$

where

$$S_V = 2(I_o)(\text{magnification})/(N_t)(L_t)(N_F)$$

where $I_o$ is the number of intercepts with the air tissue interface, $N_t$ is the number of test lines, $L_t$ is the length of the test line within the reference volume, and $N_F$ is the number of fields counted.

Alveolar wall thickness was estimated as volume per unit area of alveolar surface according to the formula $TF/S_v$, where $TF$ is the volume fraction of alveolar wall tissue.

The data from each of the three lobes of the right lung were pooled, and the means ± SD were derived for each age group. The effect of age on lung development was assessed by using a one-way ANOVA; in cases in which the data did not pass either a normality or an equal variance test, a Kruskal-Wallis one-way ANOVA on ranks was performed. Significance was accepted with a $P < 0.05$.

RESULTS

Body weight increased exponentially over the age range studied, with the highest rate of increase after 4 wk of age (Fig. 2A). Right lung volume (Fig. 2B) increased 20-fold between birth and 16 wk ($P = 0.001$), plateauing thereafter. Specific lung volume (lung volume/body wt) (Fig. 2C) decreased rapidly during the first few postnatal weeks and did not change after 4–6 wk.

Morphometric changes around birth. Between 28 dg and birth, there was an increase in the percentage of lung made up of alveolar ducts from 30 to ~50%. The volume fraction of alveoli remained at ~30% until postnatal day 3 (Fig. 3A). Both wall tissue volume in the right lung and alveolar wall thickness decreased by 40% between 28 dg and birth (Fig. 3, B and C, respectively) ($P = <0.001$). Alveolar volume increased more than twofold in the last 3 days of gestation (see Figs. 1 and 5).

Postnatal changes in morphometry. From 3 days to 1 wk, the proportion of alveolar and duct space changed, and by the second week the proportion had reversed, with ~50% of the right lung now comprising alveolar space and only 30% comprising duct space (Fig. 3A; see Fig. 1). Between birth and 9 mo of age, the percentage of tissue in the right lung was relatively constant at ~22% (Fig. 3B). After birth, wall thickness increased...
progressively (by 36% from birth) until a maximum was reached at 6 wk \( (P < 0.001) \). Between 6 wk and 9 mo of age, alveolar wall thickness dropped by 30\% \( (P = 0.005) \) (Fig. 3C).

Fig. 2. A: growth. Body weight recorded at time of death. B: lung growth. Right lung volume measured by volume displacement. C: specific lung volume. Ratio of right lung volume to body weight. Data are means ± SD for animals between 28 dg and 9 mo of age. Age*, x-axis is log\(_{10}\) of (age + 10) in days. Note that at 9 mo animals weighed 5,000+ g.

Fig. 3. A: alveolar and duct fractions. Percentage of the right lung that is made up of either alveolar (●) or duct (○) space. B: tissue fraction. Percentage of the right lung that is made up of tissue space. C: alveolar wall thickness. Alveolar wall thickness estimated for the right lung. Data are means ± SD for animals between 28 dg and 9 mo of age.
Total alveolar number (Fig. 4A) appeared to increase progressively over the age range studied (see Fig. 1) (Kruskal-Wallis ANOVA, $H = 69.966$, df = 12, $P < 0.001$). However, between 28 dg and birth, alveolar number decreased significantly ($P < 0.05$). The increase in alveolar number between 28 dg and 9 mo was described by a logarithmic equation as follows:

$$\text{Alveolar number} = -106 + 37.43 \ln(\text{age} + 18.31) \quad R^2 = 0.883$$

The rate of change in alveolar number with age (as described by the derivative of the above equation) is shown in Fig. 4B.

When standardized to body weight, alveolar number (Fig. 4C) decreased rapidly between 28 dg and postnatal week 4, thereafter remaining relatively constant ($P = 0.051$).

Average alveolar volume was quite variable at all ages and postnataally continued to increase to 16 wk of age ($P = <0.001$) (Fig. 5, Fig. 1).

Total alveolar surface area in the right lung increased progressively over the age range studied. Between 28 dg and the first week of life, surface area doubled ($P = <0.001$) and continued to increase a further 10-fold between the first week and 9 mo ($P = <0.001$) (Fig. 6A). The specific surface area (surface area/body wt) showed a similar pattern to specific lung volume and specific alveolar number, with a rapid decline between birth and 4 wk ($P = <0.001$), not changing thereafter ($P = 0.066$) (Fig. 6B). Surface area per unit volume of lung parenchyma, or surface fraction (Fig. 6C), decreased by 17% between 28 dg and 3d, after which it remained fairly uniform over the age range studied.

**DISCUSSION**

Rabbits are commonly used in respiratory research in studies modeling human lung development, premature infants, and respiratory diseases. However, little
is known about the normal pattern of lung development in this species. The aim of the present study was to comprehensively characterize the postnatal alveolar development of the rabbit to determine whether it is an appropriate model for human postnatal alveolar development.

**Lung volume.** Right lung volume (Fig. 2B) plateaued at 16 wk of age, having increased 20-fold from birth. This finding is consistent with a review by Thurlbeck and Angus (36) who reported a 26-fold increase in lung volume in humans between birth and adulthood, with the greatest rate of growth being in the first 2 yr. Interestingly, in the present study, specific lung volume (Fig. 2C) decreased rapidly between birth and 4 wk and then plateaued. The rapid decline in specific lung volume during the first postnatal month indicates that the increase in body weight greatly exceeds the increase in lung volume during this time. This is followed by a period in which the lung volume and body weight increase in proportion to one another. Burri et al. (6) found that, in rats, lung volume varies almost directly with body weight for the first 10 days of life, but thereafter lung volume increases more slowly (to the 0.7th power) than body weight.

**Lung morphometry.** The percentage of the right lung that was made up of alveolar duct decreased from 50% to 30% by 2 wk of age and remained at this proportion over the age range studied (Fig. 3A). This decrease in duct fraction reflects the bulk alveolar formation that occurred between day 3 and 2 wk of age (increase from 30 to 50% alveoli). Interestingly, duct fraction increased before it decreased (35 to 50% between 28 dg and birth, at which level it remained until almost 1 week of age) suggesting a surge in duct formation in late gestation. This is also paralleled by a significant reduction in alveolar number between 28 dg and birth (Fig. 4A).

In the present study, we found that alveoli continued to form progressively well into adulthood (Fig. 4A). Although total alveolar number increased, alveolar airspace fraction within the lung remained unchanged after 1 wk of age. Intuitively, then, the number of ducts present must also be proportionately increasing in either number and/or size. This would challenge what we know about lung development from rat studies because these suggest that ducts stop forming soon after birth and any alveolar formation beyond 2 wk of age does not involve septation of ducts but by an as yet unidentified mechanism (13). In 1950, Short (25) found that the formation of septa in rabbits ceases some time between the 10th day and the 3rd month after birth; however, this observation came from indirect and outdated measurements on perfused rather than on inflation-fixed lungs (via the trachea) at a constant pressure.

The rat has been used extensively as a model of lung development. At birth, the rat lung is predominantly made up of ducts and saccules (immature alveoli). Within a discrete 9-day period beginning 4 days after birth, bulk alveolar formation takes place (4, 5). This process is regulated by glucocorticoids and is sensitive
to exogenous glucocorticoid, as Massaro’s studies have shown (13). During this period, alveoli form via septation and elongation of secondary septa, followed by vascular maturation (5). Parallel to this is another type of alveolar formation that continues through to adulthood (13). This second type of alveolar development does not appear to be hormone sensitive and does not involve the classic processes of septation (1). It is possible that this phenomenon occurs in the rabbit because our data show that alveoli continue to increase well into adulthood.

Surface fraction (Fig. 6C) decreased slightly until 3 days, after which time it remained uniform. With lung volume and surface area increasing continually over the age range studied, this would suggest that new alveoli are continually formed to maintain an equal surface density over time. As an individual grows, the oxygen demands become greater; hence, there is a need for alveolar surface area to increase appropriately with age (Fig. 6A).

Alveolar wall thickness (Fig. 3C) showed a transient drop of 40% between 28 dg and birth, coinciding with the prenatal fall in tissue fraction (also 40%) (Fig. 3B), followed by a gradual rise to a maximum at 6 wk. This indicates that a large proportion of wall thinning occurs before birth in the rabbit. This is in contrast to rat studies, which show that alveolar wall thickening follows septation, in the second to third week after birth. The overall tissue mass is thought to decrease dramatically as secondary septa elongate to form alveoli and as the microvasculature matures (4, 5). It is possible that this phenomenon is ongoing in the rabbit because new alveoli are continually being formed, but perhaps our methods are not sensitive enough to detect these subtle changes. Early in postnatal development we do show evidence that the new alveoli that are being formed are thick walled to start with and then gradually over time. This can be seen from the changes occurring between days 3 and 7, when a surge in alveolar formation (70% increase in alveolar number) coincides with a 24% increase in wall thickness. After this time, however, the lower rate of increase in alveolar number were not associated with a detectable change in wall thickness.

Both the increase in alveolar number and the increase in alveolar size during development contribute to an increasing alveolar surface area. We found that alveolar volume increased more than twofold from 28 dg to birth and continued to increase postnaturally to 16 wk of age. The largest increase in size occurred between the first and second week (Fig. 5).

The rabbit would appear to be an appropriate model for studying the onset of alveolarization in humans. Data from our present study show that at birth rabbits have ~4.5% of the number of alveoli present at 9 mo. We found the rate of alveolar formation was highest around birth and decreased progressively to 9 mo of age (Fig. 4B). There is, however, more doubt about the suitability of the rabbit for modeling the cessation of alveolarization. Human studies have suggested that the end of alveolarization occurs anywhere between 6 mo and 8 yr of age, with Thurlbeck (35) suggesting that there is a period during which the bulk of alveoli form (0–2 yr) followed by a period of slower addition of alveoli. The data from the present study suggest a progressive increase in the number of alveoli well into adulthood. However, our aim was to determine the suitability of the rabbit as a model of alveolar development in early life, to study the effects of potential adverse influences on normal lung growth and development.

There are several limitations to the morphometric techniques we have used that need to be considered. The first is that we are extrapolating three-dimensional structures (alveoli) from two-dimensional sections. This limitation could introduce possible errors relating to differences in alveolar size. The probability of a structure appearing in any two-dimensional plane is proportional to its size whereby the larger the structure, the greater the likelihood of it appearing in an image or section (26). Our counting is therefore biased in favor of larger alveoli, and alveolar number may be overestimated in lungs with larger alveoli and conversely may be underestimated in lungs with small alveoli.

On the whole, the number of alveoli that we count per counting frame is similar (ranging from 25–35) after birth. On average we count 30 alveoli in a given area (0.35 mm²). If we overestimate this count by 5%, total alveolar number will change by 7.5% (i.e., 1.6 vs. 1.7 million). If a 10% error occurs, this will change the total alveolar number by 15% (i.e., 1.6 vs. 1.8 million). We observed that the total alveolar number increased from ~2 million to 43 million over the age range we examined; hence, errors of a few hundred thousand may change the overall magnitude of our data. However, we would not expect the overall pattern of development to change.

Furthermore, we make assumptions on alveolar shape on the basis of values derived from the mature lung, which may also lead to errors in estimates of alveolar number (37). The shape coefficient (β) relates volume to cross-sectional area and is inversely related to ε, the ratio of diameter to length (height) (37). In immature (shallow) alveoli, in which the ratio of diameter to height is large, β would be expected to be smaller. Using a shape coefficient devised for mature lungs in young animals may therefore lead to an overestimation of alveolar number. On the other hand, Randell et al. (18) measured and calculated alveolar shape constants for rats at birth, at 7 and 21 days, and after hyperoxic treatment by using the three-dimensional serial reconstruction technique and found no significant differences despite marked changes in alveolar size. This would imply a proportional growth of alveolar diameter with respect to septal height. This evidence of an alveolar shape “template” was also supported by Bianco and Frank (2), who said that alveoli are formed by a predetermined shape (close to a hemisphere) and that any enlargement with time is isotropic. Hence, there is still some debate as to the extent (if
any) that changes in alveolar shape may have on morphometric measurements. There are no published data in immature lungs either estimating an alveolar shape constant or examining the appropriateness of \( \beta = 1.55 \), and it is difficult to quantify; however, if there is a change in alveolar shape we would expect the shape constant to be smaller given that in immature (shallow) alveoli the ratio of diameter to height is larger. For example, if the shape constant were to decrease by 5%, this would translate to an increase in total alveolar number by 5%, and similarly with a 10% decrease in the shape constant used, a 10% higher total alveolar number would be calculated.

The assumption that the distribution variable \( D \) is equal to 1 may be unlikely in the developing lung given that during this time a wide variation of alveolar size would be anticipated because of the simultaneous presence of developed and developing alveoli (35).

In summary, the data from the present study suggest that rabbits may be a suitable animal model for studying the effects of potential adverse influences on lung growth in early life.

We thank Medical Research Fund of Western Australia and the Asthma Foundation of Western Australia for their financial support of this study.

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