HIGHLIGHTED TOPIC | Lung Growth and Repair

Epithelial cell distribution and abundance in rhesus monkey airways during postnatal lung growth and development

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The lungs undergo a period of lung growth and maturation that occurs both before and after birth. While the lung grows in size, many of the epithelial cells also mature and differentiate. The early postnatal period of lung development is of particular interest from a human health perspective for two reasons: 1) this period of lung development may encompass several “windows of susceptibility” for exposure to environmental pollutants (20, 22), and 2) several common and debilitating human lung diseases are initiated in early childhood (i.e., cystic fibrosis and asthma) (11, 17, 29). The nonhuman primate lung is ideal for the study of airway epithelial biology because it contains a large range of epithelial cell phenotypes in a complex multilayered structure in both proximal and distal bronchi, similar to humans. Furthermore, rhesus macaques undergo substantial postnatal lung maturation and growth, as do humans. For this reason several animal models are now being developed in nonhuman primates to study human airway diseases that have their origins in early childhood (11, 17, 29). To more fully understand the impact of the development process in these new animal models of lung disease requires a better appreciation of the dynamics of the epithelial populations that line the airways. Previous studies have described in detail the cellular events associated with fetal lung morphogenesis in humans, particularly in relation to the postnatal development of the gas-exchange area (2, 3, 16). Whereas there is abundant information on airway epithelium in a number of rodent species used to model asthma (8, 27), there are few quantitative data on normal postnatal development of airway epithelium in most species. It is also important to point out that there is a basic difference in airway epithelial biology between primates and rodents. Primates have mucous cells present and differentiating as part of the normal steady-state airway epithelium in the postnatal lung. Rodents do not normally have mucous cells in the intrapulmonary airways. Detection of these cells in the airways of rodents developed to study allergic airway disease likely involves different regulatory pathways and differentiation processes (metaplasia) than the formation of excess mucous cells in an epithelial environment that normally has them. One goal of our study was to provide quantitative information on the postnatal maturation of conducting airway epithelial cells in a species (rhesus monkeys) in which the predominant airway secretory epithelial cell is the mucous goblet cell.

As the airways grow in both length and diameter, the epithelial surface area increases greatly. For example, a 50% increase in both tracheal diameter and tracheal length results in a greater than twofold increase in basement membrane surface area because surface area increases as the product of the circumference and the length. We recently showed that the rhesus basement membrane zone develops between 1 and 6 mo of age (10). Pre- and postnatal maturation of tracheal conduct-
The axial path airway tree was exposed by microdissection and the airway branches noted. This enabled sampling of the axial airway path at defined airway regions. Samples were stored in fixative at 4°C until use. Samples were postfixed in 1% osmium tetroxide in Zetterquist’s buffer, processed by large block methodology, and embedded in Araldite 502 epoxy resin. Specimens were sectioned at 1 µm, stained with 1% toluidine blue in 1% sodium borate solution, and imaged on an Olympus BH-2 microscope in brightfield mode. We examined rhesus monkey lungs from the following postnatal ages: 4–6 days (n = 3–7), 1 mo (n = 3), 2 mo (n = 5), 3 mo (n = 3), and 6 mo (n = 5–6).

**Quantitative histopathology.** The abundance of normal airway epithelial cells was analyzed by use of high-resolution images (magnification ×60 or greater) and morphometric procedures previously used in (25) and discussed in detail by Hyde et al. (15). All the measurements were made using 1.0 µm resin sections. Three airway generations were counted: 1) the trachea, 2) the intrapulmonary bronchi between the first and second branches of the right middle lobe, and 3) the intrapulmonary bronchi between the sixth and eighth branches of the right middle lobe. The entire circumference of each defined airway generation was imaged at ×60 magnification, and a minimum of 10 fields were sampled for counting with a random number table and a uniform random sampling scheme (14). The volume densities (Vv) of six categories of cells (basal, goblet, nonciliated, ciliated, dark ciliated, and leukocytes; see Fig. 3) were defined by point (P) and intercept (I) counting of bronchial epithelial vertical profiles using a cycloid grid and Stereology Toolbox (Morphometrix) on collected images. Vv was calculated by the formula

\[ V_v = P_v = P_l/P_t \]

where Pp is the point fraction of P, the number of test points hitting the structure of interest, divided by Pt, the total points hitting the reference space (epithelium). The surface area of epithelial basement membrane per reference volume (Sv) is determined by point and intercept counting and calculated by using the formula

\[ S_v = 2L_v/L_e \]

where L1 is the number of intersections with the object (epithelial basal lamina) and L2 is the length of the test line in the reference volume (epithelium). The thickness of the epithelium, or volume per unit area (Vv) of basal lamina (µm²/µm³), was calculated by using the formula for arithmetic mean thickness (τ):

\[ \tau = V_v/S_v \]

Measurements of tracheal diameters were made from the epithelial surface of the long and short diameters of the airway rings stained with toluidine blue (10). The average diameter was determined and used to calculate circumference. Airway lengths were measured directly in microdissected whole lung, noting the distance between airway branches of the main axial path by using calibrated eyepiece micrometer mounted on a stereomicroscope (Wild Heerbrug, Switzerland).

**Counting criteria.** Basal cells were defined as cells found along the basement membrane, nucleus adjacent to basement membrane, generally darker than ciliated cells. Ciliated cells contained cilia and were light staining. Goblet cells were dark-staining tall columnar cells with mucous granules. Leukocytes were cells with a clear to very light cytoplasm and an irregular nucleus (within the epithelium only, usually small cells). Dark ciliated cells are dark-staining tall columnar cells with cilia. Undefined is an all inclusive category that included most cells that could not be clearly characterized by the criteria above (i.e., intermediate basal cells, portions of cells, columnar cells lacking cilia or granules, dividing cells, and cells that were similar to basal cells in color but were in the middle of the epithelium). Sections from airways that were mechanically disrupted by sample processing were not used.
Statistics and calculations. Increases in airway surface area, estimated airway volume, and cross-sectional area were calculated from the mean circumference of airway generations 1–8 and the total (cumulative) measured length of these airway generations by using formulas that assume a cylindrical shape of the airway. Tissue mass \(V_s\) and arithmetic mean thickness were calculated per animal from counts made from at least 10 fields/airway and were used to calculate the mean and SD for each group. Differences between groups of values were determined by ANOVA and one-way regression analysis. Determination of significance between treatment groups of one age was based on post hoc analysis with Scheffé’s \(F\)-test with significance set at \(P < 0.05\). Comparisons between two different ages of animals for the same treatment were made using Student’s \(t\)-test.

RESULTS

Postnatal animal growth. Infant rhesus monkeys increase significantly, 2.8-fold, in body weight (Fig. 1A) during the postnatal period from 4–6 days to 6 mo of age. As the body grows, the fixed lung volume of the right middle lobe increases

![Fig. 1. A: body weight of infant monkeys was significantly smaller than that of 6-mo animals with a >3-fold increase in weight over this time period. B: the volume of the fixed right middle lobe was significantly smaller in the infant monkeys than in the 6-mo animals. C: the total length of intrapulmonary airways that were sampled for morphometric quantitation were measured. Infant monkeys had significantly less airway length compared with 6-mo monkeys. D: the 2 largest airway generations, trachea and intrapulmonary generation (Gen) 1–2, increased significantly in circumference with age. *Significantly different vs. 6-mo animals at \(P < 0.05\). E: the fold increase over time from 5 days to 6 mo of age is shown for body weight, volume of the fixed middle lung lobe, cumulative length of airway generations 1–8, and mean fold increase in circumference for the sum of trachea, generations 1–2, and generations 6–8. Fold increase in mean airway basement membrane surface area was calculated from the cumulative length and mean circumference of generations 1–8. Change in volume of airway was calculated for cumulative airway generations 1–8. F: fold increase in airway cross-sectional areas by airway generation. Airway cross-sectional area was estimated on the basis of tracheal diameters measured from basement membrane to basement membrane in lungs inflation fixed at constant pressure.](image-url)
greater than 2.3-fold as well; from a mean fixed lung volume of 3.54 ± 0.43 ml to a volume of 8.10 ± 1.46 ml (Fig. 1B). As the lung en toto grows in size, some of the increase is due to the conducting airways increasing in both length and circumference (Fig. 1, C and D). We measured the cumulative length (Fig. 1C) of the intrapulmonary conducting airways from intrapulmonary airway generation 1 to generation 8 (length was unavailable for trachea) used in this study. Cumulative length was significantly increased 1.2-fold from 4–6 days to 6 mo of age. However, airflow lengths of individual airway generations were not significantly different (data not shown).

The circumference of specific airways was estimated from airway cross sections and increased significantly with age for the two most proximal airway generations, the trachea (1.3-fold) and the proximal intrapulmonary bronchus (1.4-fold, Fig. 1C). The circumference of the distal bronchus did not increase with age. The fold increase from 5 days to 6 mo in various airflow parameters is illustrated in Fig. 1, E and F. Body weight increased the most. Mean circumference for all three airway generations changed the least. Mean circumference for all three airflow generations changed the least. Mean cross-sectional area changed the most in airway generations 1–2. The age-dependent increase in airway cross-sectional area was only significant for the trachea.

Quantitative histology. High-resolution light micrographs of the conducting airway epithelium of the trachea, large bronchi (generations 1–2 of intrapulmonary airways), and small bronchi (generations 6–8 of intrapulmonary airways) were examined during postnatal development at 4–6 days and 1, 2, 3, and 6 mo of age. Figure 2 compares the histology of these three airway generations from the youngest animals examined, 4–6 days old, with that of the oldest, 6 mo old. As shown in Fig. 3A, tall columnar ciliated, dark ciliated cells, mucous goblet cells, as well as basal cells and leukocytes were located in the airway epithelium of all airway generations in all animals. Cells containing mitotic figures in the large bronchi were noted, predominantly at ≤1 mo of age, and were located primarily in the basal cell layer (Fig. 2E). Consistent with previous studies from this group (10), we observed that the basement membrane zone changed organization, becoming more discrete, with age in the large airways (compare Fig. 2C with 2D). We also characterized a rare cell type (4% of the epithelium by volume) that was found in all airway levels and that contained a ciliated surface, but was darker staining than other ciliated cells (shown in Figs. 2, C and F, and 3A). We called this cell type “dark ciliated.” These cells were found among the tall columnar ciliated and mucous cells and were found both in clusters and singly. These cells frequently contained clear cytoplasmic inclusions in the upper half of the cell above the nucleus. These cells did not appear to have degenerating or fragmented nuclei.

Quantitation revealed that the thickness of the airway epithelium was unchanged with age in the trachea and the large bronchi (Fig. 3B). However, epithelial thickness decreased significantly (1.75-fold) in the small bronchi when 6-mo animals were compared with 2-mo-old monkeys. Total ciliated cell mass (the sum of dark ciliated plus normal ciliated) decreased significantly (2-fold) between 1 mo and 6 mo. Dark ciliated cells were a small percentage of the ciliated cell total (Fig. 4, B–D), but were not significantly different in mass by airflow level or age. Mass (Vv) of basal, undefined and leuko-

Fig. 2. High-resolution light micrograph of representative airway epithelium from a 5-day-old monkey (A, C, E) and a 6-mo-old monkey (B, D, F). Three airway generations are illustrated trachea (A and B), proximal bronchus (C and D), and distal bronchus (E and F). The airway epithelium contained ciliated, mucous goblet, and basal cells in abundance but total epithelial thickness decreases as the airways decrease in size. Dark ciliated cells (DC) were infrequent. Cells with mitotic figures (arrowhead) were more common in younger animals. The basement membrane became more distinctive with age (arrows). Bar in F is 30 μm.
cyte cell types were unchanged with age (Fig. 5, A, C, and D). In the trachea, goblet cells decreased significantly with age with 6-mo animals significantly different from the 5-day-old animals (Fig. 5B). Ciliated cells had the largest mass followed by basal cells, goblet cells, and “undefined” cells. In all airway levels, leukocytes and dark ciliated cells were infrequently detected. The volume fraction (Vv) of epithelium occupied by various cell types did not vary significantly by age in any airway level (Figs. 6 and 7). The Vv of all ciliated cells (Fig. 6A) increased significantly with decreasing airway size for the following ages: 5 days, 3 mo, and 6 mo (P < 0.05 by ANOVA for each age). Although there was a trend for the Vv of all ciliated cells to increase with decreasing airway size this was not significant for the 1- and 2-mo-old animals. The Vv of dark ciliated cells did not vary significantly by age or airway level (Fig. 6, B–D).

DISCUSSION

This study is the first to quantitatively define the postnatal pattern of cell distribution and abundance of the proximal conducting airway epithelial cells of the rhesus monkey, a species with mucous cells, while simultaneously defining changes in airway size. As part of this study, we defined changes in airway length and circumference and found, as expected, significant increases in both parameters with age. We found that as basement membrane surface area increased with increasing age, the epithelial cell organization changed little.
from 5 days to 6 mo of age. Tracheal cell mass was present at nearly constant levels of thickness in the postnatal period; thickness at 5 days in the trachea is not different from thickness at 6 mo. Airway epithelial thickness is generally thought to increase with increasing airway size and age (9). However, mean epithelial thickness decreased in the distal bronchi at 6 mo of age compared with distal bronchi in younger ages. This may be due to the airways in this study increasing markedly in length and diameter at this age and so epithelial thickness decreases as cell numbers spread out to cover an increased surface area.

It is important to note that in this study the airways were sampled at defined airway generations between branch points. Branch points have been reported as loci for epithelial cell proliferation and replacement after airway injury in mice (28). However, we do not know whether this same mechanism occurs in animals that have pseudostratified airway epithelium such as primates. If the branch points served as loci for proliferation and growth, this would explain why we do not see shifts in cell populations with age in the airway sites we sampled (such as increases in basal cells or undefined cells) in this study.

Dysanaptic growth is uneven lung growth between compartments and is frequently used to refer to a pattern where airway growth lags behind parenchymal growth. This has been found to occur in studies of compensatory lung growth after pneumonectomy (5, 7) and has been used to explain inequalities in expiratory flow rate and lung volume (1, 12). Whereas the airway branching structure is thought to be established primarily in the fetal period, airway growth in both length and diameter also occurs as animals become larger in the postnatal period. In contrast to the conducting airways, alveolar morphogenesis occurs largely postnatally through septation (6). Septation occurs between 4 and 14 days of postnatal age in rats (4, 6). Our present study shows that airway growth (primarily lengthening) also occurs in the postnatal period and continues both through and beyond the most exponential phase of formation of new alveoli (4, 6). In the rat, alveolar surface area undergoes a phase of rapid growth from 4 to 21 days postnatal and increases more than sixfold during this period. Lung volume increases ~2.5-fold simultaneously. After 21 days, alveolar surface area increases at a slower rate. If this pattern of alveolar growth also occurs in the postnatal period in monkeys, then the increases in basement membrane and airway cross-sectional area lag behind that of the alveoli and do not increase to the same extent.

Our studies also show that airway length increases to a greater degree than circumference in the first 6 mo after birth in the rhesus monkey. On the basis of the equation for airway resistance (Raw) where Raw is inversely proportional to the...
airway radius raised to the fourth power and directly related to airway length, length has less of an influence on changing resistance than diameter. In this case a large increase in length as well as a significant increase in circumference with normal lung development would likely be driven by the greater influence of circumference and would predict a corresponding moderate decrease in Raw. Regarding growth of the airways, our measurements show that airway length and circumference increase at a slower rate than both lung volume and body weight. However, when estimated axial path airway volume is compared with lung lobe volume, the rate of increase over time is very similar (2.23 vs. 2.29-fold). However, airway cross-sectional area increased most in generation 1–2, twofold, during this time. Further studies are needed that measure the entirety of the conducting airways to define the relationship between the gas-exchanging alveoli, both the proximal and distal conducting airways, and the parenchyma during postnatal lung growth in the rhesus monkey.

Our results in the 6-mo animals were comparable with a previous study in 3-yr-old “adult” rhesus monkeys (23) for epithelial thickness for both proximal bronchus (6 mo $16.7 \pm 4.9$; adult $21.7 \pm 4.0 \, \mu m^2/\mu m^3$) and distal bronchus (6 mo $11.9 \pm 2.7$; adult $16.1 \pm 2.7 \, \mu m^2/\mu m^3$). However, tracheal thickness was markedly larger in the adult animals ($46.6 \pm 13.9 \, \mu m^2/\mu m^3$) compared with the 6-mo monkeys in the present study ($26.8 \pm 4.8 \, \mu m^2/\mu m^3$). Much of this increase is due to increased amounts of mucous goblet cells in the large airways of the adult animals; mass was $14.1 \pm 3.4 \, \mu m^3/\mu m^2$. In comparison, the 6-mo-old monkeys had a mucous cell mass of $3.2 \pm 0.9 \, \mu m^3/\mu m^2$, greater than fourfold less. This could indicate that mucous goblet cells undergo continued maturation after 6 mo postnatal age. However, the adult animals in the previous study (23) were “field” controls that underwent a 2-wk period of acclimation in filtered air before necropsy. This means that they were housed outdoors in the ambient environment of the Sacramento region for most of their lives. Hence, the values previously reported for these adult animals likely reflect their cumulative environmental exposures to air pollution. This concept is further supported by the fact that if the differences in mucous cell abundance were entirely due to postnatal maturation continuing after 6 mo of age, we would expect to see proportionally greater increases in mucous cells in the adult bronchi than in the trachea, because airway cell maturation occurs in a proximal-to-distal direction (26). This is not the case. Although mucous cell mass is indeed larger in the bronchi of the adult animals compared with 6-mo animals, it is not increased to the extent that it is increased in the trachea. This argues for a contribution of air pollution encountered in housing as affecting the adult airway thickness and mucous goblet cell abundance. Airway mucous cells in the previous study may also increase if there is a disease process. The fact that intraepithelial leukocytes, basal cells, and ciliated cells are all found at similar levels in both studies also argues against an acute infectious agent of some sort affecting the results in the previously reported adult animals (23) and confirms the reproducibility of our technique. This points out a strength and a weakness of our present study: what we have established is the pattern of airway epithelial cells for animals that are housed entirely in air free of ozone, pollen, and dust. Obviously, normal postnatal lung maturation in the “native” environment includes exposures to dusts, allergens, and pollutants that our monkeys did not have in this study. Regardless, we have defined the distribution and abundance of airway epithelial cells in animals housed in a very clean environment.

We defined a rare cell type in this study: dark ciliated cells (<4% of the total epithelium by volume). They were a distinct...
category of columnar cells characterized by a dark cytoplasm with apical cilia and small clear apical inclusions in some cells. These cells were found both singly and in clusters. In their frequency, this cell type resembles the PGP 9.5-positive cells described in a recent study of nerve elements in rhesus monkey epithelium (18). However, the dark ciliated cells did not contain lateral projections nor were they found directly anchored to the basement membrane as described for the PGP 9.5-positive cells in the study by Larson et al. (18). Another possibility is that these cells are apoptotic. Apoptotic cells have been shown to stain darkly. However, we did not see any degradation or condensation of the nuclei in these cells. The columnar shape and location in the apical portion of the airway epithelium as well as their description as containing both cilia and clear apical inclusions (small granules) makes it more likely that the dark ciliated cells are a correlate to the "intermediate" cells described in previous histopathology studies of monkey airways (32). These cells are thought to represent an intermediate cell type that may be a precursor to either ciliated cells or mucous goblet cells. In this case it would be logical to find these cells in some abundance in the airway epithelium of these animals during postnatal lung growth and development.

The principal finding from this study is the constancy of various epithelial cell proportions in the airway epithelium of these rapidly growing animals. If each airway is considered a simplified tube, then the increase in basement membrane surface area required to anchor the epithelial tube as it changes between 5 days and 6 mo of age is at least an increase of 1.6-fold. This means that the airway epithelium both expands in cell number but also maintains fairly constant levels of differentiated cells in spite of a rapidly increasing surface area. We found that mucous cell abundance in 6-mo postnatal animals is fourfold less than that previously reported for adult rhesus monkeys (23). Furthermore, our results indicate that airway cells in different proximal airway generations differentiate similarly. The distribution and abundance of most airway cells did not change with age. However, in the most distal airways examined there were decreases in mass of ciliated cells, and in the trachea, goblet cells were significantly decreased at 6 mo of age. The fact that the volume fraction (%) of cell types did not vary with age implies a mechanism for tight control of cell types as well as their distribution and abundance in the airways. We speculate that the disruption of this tight control results in human airway diseases that have their origins in early childhood.

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