Increased epithelial cell proliferation in very premature baboons with chronic lung disease

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Received 5 February 2002; accepted in final form 15 June 2002

Maniscalco, William M., Richard H. Watkins, Michael A. O'Reilly, and Colleen P. Shea. Increased epithelial cell proliferation in very premature baboons with chronic lung disease. Am J Physiol Lung Cell Mol Physiol 283: L991–L1001, 2002. First published June 28, 2002; 10.1152/ajplung.00050.2002.—Coordinated proliferation of lung cells is required for normal lung growth and differentiation. Chronic injury to developing lung may disrupt normal patterns of cell proliferation. To examine patterns of cell proliferation in injured developing lungs, we investigated premature baboons delivered at 125 days gestation (E) and treated with oxygen and ventilation for 6, 14, or 21 days (PRN). Each PRN treatment group contained 3 or 4 animals. During normal in utero lung development, the proportion of proliferating lung cells declined as measured by the cell-cycle marker Ki67. In the PRN group, the proportion of proliferating lung cells was 2.5–8.5-fold greater than in corresponding gestational controls. By 14 days of treatment, the proportion of cells that expressed pro-surfactant protein B (proSP-B) was ~2.5-fold greater than in gestational controls. In the PRN group, 41% of proliferating cells expressed proSP-B compared with 5.8% in the gestational controls. By 21 days of treatment, proliferation of proSP-B-expressing epithelial cells declined substantially, but the proportion of proliferating non-proSP-B-expressing cells increased approximately sevenfold. These data show that the development of chronic lung disease is associated with major alterations in normal patterns of lung-cell proliferation.

NORMAL LUNG DEVELOPMENT REQUIRES highly ordered proliferation and differentiation of pulmonary epithelial and mesenchymal cells. Injury to developing lungs may alter the normal patterns of cell proliferation and contribute to the disordered architecture and cell function that are characteristic of chronic lung disease (CLD) in premature newborns. Bronchopulmonary dysplasia (BPD) is a CLD of premature infants that results in prolonged hospitalization and substantial morbidity. The pathophysiology of BPD involves oxidant injury, baro/volutrauma, and disordered lung repair that result in poor alveolization and disrupted microvascular development (10).

Few studies have examined normal patterns of cell proliferation in developing lungs. Adamson and King (3) found that total cell proliferation declined during fetal rat lung gestation. The relative rate of epithelial proliferation decreased during the latter stages of gestation, whereas endothelial cell proliferation increased during this time. Differentiation of alveolar epithelial cells from type II to type I was associated with decreased epithelial cell proliferation. The regulation of cell proliferation in lung development is largely unknown, but mice that lack corticotropin-releasing hormone have increased epithelial cell proliferation compared with wild-type mice, which suggests a role for endogenous glucocorticoids in mediatiation of the normal gestational decrease in cell proliferation (26).

Increased cell proliferation, particularly of alveolar type II epithelial cells, is characteristic of several acute and chronic pulmonary injuries. For example, adult primates treated with 100% oxygen and ventilation for 96 h had an increased number of type II cells (30). Similarly, type II cell numbers increased in adult animals of several species that were exposed to 100% oxygen and allowed to recover in air for 7 days (34). Adult baboons exposed to 60% oxygen for 14 days had increased type II cell numbers, which decreased during recovery in air (17). Adult humans with fibrotic lung disease often have increased numbers of cuboidal epithelial cells in the distal air spaces (21). These studies suggest that an increased quantity of type II cells may be central to repair of the alveolar epithelium after injury from agents such as oxidant gases (18). The mechanisms of increased distal cuboidal epithelial cells in chronic lung injuries have not been defined.

Less is known about proliferation of distal air space epithelial cells in developing lungs subjected to acute or chronic injury. Newborn mice exposed to 100% oxygen for 7 days had increased numbers of type II cells (7). A transient increase in overall cell proliferation was noted in newborn mice exposed to 85% oxygen for 28 days (37). Premature primates treated with venti-
lation and 80–100% oxygen had increased volume density of type II cells (13). Some premature human infants with BPD had increased numbers of cuboidal epithelial cells in distal saccules (10). Proliferation of type II cells as a mechanism of increased type II cell number in injured fetal lungs has not been examined.

In developing lungs, alterations in the proliferation or phenotype of the distal air space epithelium may affect lung development. The decreased alveolarization that is found in newborn mice treated with 85% oxygen and in patients with BPD may be related to altered epithelial cell proliferation (10, 37). Other defects in lung development may result from an abnormal distal epithelial cell phenotype. For example, fetal distal air space epithelial cells express vascular endothelial epithelial cell phenotype. For example, fetal distal air space epithelial cells in distal saccules (10). Proliferation of infants with BPD had increased numbers of cuboidal epithelial cells, which may contribute to disruption of lung structure and function.

Thus injury-induced alterations in distal epithelial cell proliferation may affect multiple components of lung development.

The present study investigated cell proliferation in extremely premature baboons that developed CLD after treatment with oxygen and ventilation for ≈21 days. Compared with gestational controls, these animals had increased overall cell proliferation and increased numbers of epithelial cells that expressed proSP-B. Epithelial cell proliferation was increased at 6 and 14 days of treatment but declined by 21 days. These data suggest that development of CLD in premature fetal primates results in altered patterns of distal air space epithelial cell proliferation, cell number, and phenotype, which may contribute to disruption of lung structure and function.

METHODS

Animals. Lung samples from prematurely delivered baboons were provided by the Southwest Foundation for Biomedical Research (San Antonio, TX). All animal procedures were reviewed and approved to conform with American Association for Accreditation of Laboratory Animal Care guidelines. Normal fetal baboons [gestational controls (GC)] were delivered by elective hysterotomy at 125, 140, 146, 160, and 175 days gestation (125 GC, 140 GC, 146 GC, 160 GC, and 175 GC, respectively); term is 185 days. Birth weights, the number of animals in each group, and other characteristics are listed in Table 1. To study the effects of oxygen and ventilation, some 125-day-gestation animals were intubated, given exogenous surfactant (Surfactant, 4 ml/kg, Abbott Laboratories), and maintained with ventilator support and oxygen to achieve normal blood-gas measurements for 6, 14, or 21 days (PRN groups: 6dPRN, 14dPRN, and 21dPRN, respectively). Details of ventilatory management and pulmonary function data in this model have been reported (14, 38). Ventilator settings for the animals used in this study are summarized in Table 1. The 6dPRN and 14dPRN animals received only parenteral nutrition (Trophamine, Braun Medical), whereas the 21dPRN animals were started on enteral feedings at 7 days of age (Similac, Abbott Laboratories). The 140 GC and 146 GC animals were controls for the 14dPRN and 21dPRN animals, respectively. All animals were treated with antibiotics, and none had overt signs of infection. At the time of necropsy, the right lower lobe was removed and fixed with intrabronchial phosphate-buffered 4% paraformaldehyde at 20 cmH2O of pressure for 24 h. As detailed by Coalson et al. (14), the lobe was cut into three serial, equally spaced horizontal sections, which were embedded in paraffin and cut into 4-μm sections. Separate lung sections from each animal were evaluated for histone H3 or surfactant protein C (SP-C) by in situ hybridization, Ki67 by single immunohistochemistry (IHC), and both Ki67 and proSP-B by dual fluorescence IHC as detailed (see Immunohistochemistry).

In situ hybridization. For histone H3, sense and antisense 33P-labeled riboprobes were synthesized using a 553-bp mouse histone H3 cDNA template (28). The SP-C cDNA was obtained from Jeffery Whitsett (University of Cincinnati). In situ hybridizations were performed as described previously (25) with the following modifications: sections were prehybridized for 3 h and hybridized for 16 h at 53°C. After rinses in 2× saline sodium citrate (SSC) and digestion with RNase A, the sections were rinsed in 2× SSC again and in 0.1× SSC for 30 min at 64°C. The slides were dipped in a 1:1 dilution of NTB-2 emulsion (Eastman Kodak, Rochester, NY) and exposed for 4 days before developing.

Immunohistochemistry. Primary antibodies were mouse anti-human Ki67 (clone Ki-S5, DAKO, Carpenteria, CA) and rabbit anti-human proSP-B (AB3430, Chemicon International, Temecula, CA). Secondary antibodies were biotinylated horse anti-mouse IgG (Vector Labs, Burlingame, CA), Texas red-conjugated donkey anti-mouse IgG, and FITC-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Labs, West Grove, PA).

### Table 1. Characteristics of gestational controls and animals treated with oxygen and ventilation

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Postconceptional Age at Death, days</th>
<th>Birth Wt, g</th>
<th>Autopsy Wt, g</th>
<th>Maximum FIO2, cmH2O</th>
<th>Maximum PIP, cmH2O</th>
<th>Ventilator Rate, breaths/min (range)</th>
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<tbody>
<tr>
<td>125 GC</td>
<td>4</td>
<td>125</td>
<td>363 ± 37</td>
<td>355 ± 63</td>
<td>0.48 ± 0.15</td>
<td>24 ± 3</td>
<td>31–46</td>
</tr>
<tr>
<td>6dPRN</td>
<td>4</td>
<td>131</td>
<td>376 ± 70</td>
<td>377 ± 78</td>
<td>0.49 ± 0.14</td>
<td>24 ± 2</td>
<td>27–54</td>
</tr>
<tr>
<td>140 GC</td>
<td>4</td>
<td>140</td>
<td>499 ± 76</td>
<td>402 ± 66</td>
<td>0.47 ± 24</td>
<td>24 ± 3</td>
<td>21–75</td>
</tr>
<tr>
<td>14dPRN</td>
<td>4</td>
<td>140</td>
<td>416 ± 68</td>
<td>394 ± 37</td>
<td>0.47 ± 24</td>
<td>24 ± 3</td>
<td></td>
</tr>
<tr>
<td>160 GC</td>
<td>3</td>
<td>160</td>
<td>804 ± 21</td>
<td>804 ± 21</td>
<td>0.47 ± 24</td>
<td>24 ± 3</td>
<td></td>
</tr>
<tr>
<td>175 GC</td>
<td>3</td>
<td>175</td>
<td>993 ± 85</td>
<td>993 ± 85</td>
<td>0.47 ± 24</td>
<td>24 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of baboons. Experimental animals were delivered at 125 ± 2 days gestation and treated with oxygen and ventilation for 6 (6dPRN), 14 (14dPRN), or 21 (21dPRN) days. Gestational controls (GC) delivered at 125, 140, 146, 160, and 175 days (term is 185 days). FIO2, fraction of inspired O2; PIP, peak inspiratory pressure.
After paraffin was removed, the lung sections were rehydrated through graded ethanol washes and rinsed in deionized water. When peroxidase detection was used, slides were treated with 1.5% hydrogen peroxide in methanol and rinsed in water. Antigen retrieval was performed by heating the slides in 50 mM Tris-HCl at pH 10 for 14 min in a microwave oven and then cooling them at 25°C for 20 min. Slides were rinsed in 50 mM Tris-HCl with 150 mM NaCl at pH 7.5 (Tris-buffered saline (TBS)) and blocked with 5% serum in TBS with 0.05% Tween 20 (TBST).

For double-fluorescence IHC, slides were incubated overnight at 4°C with two primary antibodies diluted in TBSST with 5% donkey serum (Ki67, 1:50 dilution; anti-proSP-B, 1:2,000 dilution). The slides were then rinsed in TBSST and incubated with the appropriate fluorescent secondary antibodies diluted 1:100 in TBSST with 3% donkey serum. Slides were again rinsed in TBSST, counterstained with 4’,6-diamidino-2-phenylindole (DAPI) nucleic acid stain, rinsed in TBS, and mounted with Vectashield mounting medium (Vector Labs).

For IHC using dianinobenzidine (DAB) as the enzyme substrate, slides were incubated overnight in a 1:50 dilution of anti-Ki67 antibody in TBS with 5% horse serum, rinsed in TBS, incubated with biotinylated horse anti-mouse IgG, rinsed, treated for 30 min with Vectastain ABC Elite, rinsed, and stained with DAB substrate with nickel (Vector Labs) for 2 min.

**Quantification of fluorescence IHC.** Random, noncontiguous fields of lung parenchymal/distal air space sacculles were acquired using the ×40 objective of a Nikon E-800 fluorescence microscope and a SPOT RT camera. The fields were selected using the DAPI image to prevent bias toward fields with the Texas red or FITC signals. For the GC animals, 3–5 fields per lung section were obtained from 3 or 4 separate animals at each gestational age. For the PRN animals, 6 fields per lung section were obtained from 3 or 4 separate animals in each group. Fields that contained a large airway or blood vessel were rejected. One slide was analyzed for each animal. Different fluorescence filters were used to acquire images of each field displaying all nuclei (DAPI), proliferating cells (Texas red), and proSP-B (FITC). The images were merged to identify cells that were positive for Texas red or FITC. Quantification was performed using Metamorph (Universal Imaging, Downingtown, PA). All nuclei were counted by using the DAPI image and configuring Metamorph to measure total nuclei based on the average area of a nucleus. Each field contained 300–800 nuclei (486 ± 135, mean ± SD). Proliferating cells (Texas red positive), proSP-B-positive cells (FITC positive), and cells positive for both Texas red and FITC were counted manually by using Metamorph to mark the counted cells. For each animal, the counts from all of the fields were summed and the following ratios were determined: proliferating/total cells; proSP-B-positive/total cells; proliferating proSP-B-positive/total cells; proliferating non-proSP-B-positive/total cells; proliferating proSP-B-positive/proSP-B-positive cells; and proliferating proSP-B-positive proliferating cells. The ratios for all animals at each time point were averaged.

**Statistics.** For data that showed changes during gestation, the statistical difference was calculated using the Kruskal-Wallis test. The Wilcoxon rank-sum test was used to compare data between PRN and GC animals. Calculations were performed using Stata software. A value of $P < 0.05$ indicated statistically significant differences.

**RESULTS**

**Ki67 immunostaining decreases in baboon lung development.** Ki67 is a nuclear protein that is expressed in all active phases of the cell cycle (G1, S, G2, and M) but not in G0 (32). Although its function is not known, Ki67 expression is required for cell proliferation. Because it has a relatively short half-life (90 min), Ki67 marks proliferating cells rather than cells that have just left the cell cycle. Ki67 is not expressed during DNA repair (20), which is an important consideration, because oxidants may lead to DNA damage and unscheduled DNA synthesis (27). Immunostaining for Ki67 in fetal baboon lung at 125 days gestation showed a substantial number of nuclei that contained this protein (Fig. 1A, arrow). The immunostained nuclei were found in a broad range of cell types including distal air space epithelial, interstitial mesenchymal, airway epithelial, and smooth muscle cells. However, between 125 and 175 days gestation (term is 185 days), the number of immunostained nuclei appeared to decrease (Fig. 1, B–D). No distinctive cell pattern in the decrease in Ki67 immunostaining was noted. Overall, these data suggest a decline in the rate of cell proliferation during this stage of lung development.

**Ki67 immunostaining increases in CLD animals.** Fetal baboons delivered at 125 days gestation were treated with surfactant and maintained on supplemental oxygen and ventilation for either 6 or 14 days to achieve normal blood gas levels. The lung morphology after both 6 and 14 days was abnormal. The 6dPRN animals had abundant inflammatory cells in distal air spaces (Fig. 2A). Both time points had atelectatic regions and simplified distal air space sacculles (Fig. 2, A and B). Some lung areas, however, appeared relatively normal (not shown). Compared with the 140 GC animals (Fig. 1B), the 14dPRN animals (Fig. 2B) had hyperexpanded distal sacculles that lacked secondary crests. Compared with GC groups, PRN groups had an increased number of nuclei that immunostained for Ki67. Although immunostained nuclei in interstitial mesenchymal cells were prominent, substantial numbers of distal air space epithelial cells had nuclei with Ki67 (Fig. 2, A and B, arrows). These findings were somewhat heterogeneous, and some regions in both PRN groups had relatively few proliferating cells (not shown). These data indicate that high rates of cell proliferation are maintained in many areas of CLD lung and that distal sacculle epithelial cells may have a particularly high rate of proliferation.

**Expression of histone H3 in lung development and CLD.** To corroborate our observations on Ki67 expression, we performed in situ hybridization for histone H3, another proliferation marker. Histone H3 mRNA expression begins in late G1 phase, increases 20–100-fold in S phase, and is rapidly degraded at the end of S phase (19, 22). Similar to Ki67, histone H3 is not expressed in DNA repair. Histone H3 in situ hybridization showed decreased expression of this marker between 125 and 140 days gestation (Fig. 3, A and B), which continued until at least 175 days gestation (not
shown). In both PRN groups, some areas of lung had histone H3 expression that appeared greater than in the GC groups (Fig. 3, C and D). Higher power views indicated that some of the histone H3-expressing cells were distal air space epithelial cells and others were interstitial cells (not shown).

Cell-specific proliferation in lung development. To quantify changes in cell proliferation and identify proliferating epithelial cells, we used DAPI to mark all cell nuclei and combined with this double immunofluorescence for Ki67 and proSP-B. DAPI yields a blue color (Fig. 4A, yellow arrow); Ki67 (Texas red) presents with a magenta color when combined with DAPI (Fig. 4A, dashed arrow); and proSP-B (FITC) indicates with a green color (Fig. 4B, solid arrow). The orange color is autofluorescence of red blood cells. Between 125 and 175 days gestation, the proportion of cell nuclei that expressed Ki67 appeared to decrease, and the proportion of cells that stained for proSP-B appeared to increase (Fig. 4). The staining intensity for proSP-B in individual cells also increased. Quantifying these data by image analysis (Fig. 5A) showed that the proportion of Ki67-positive nuclei decreased from 5.2 ± 0.5% of total nuclei at 125 days gestation to 0.17 ± 0.01% at 175 days gestation (P < 0.05). The proportion of total peripheral lung cells that expressed proSP-B increased twofold between 125 and 175 days gestation (P < 0.05; Fig. 5B). These data suggest decreased cell proliferation and accumulation of proSP-B-expressing cells during this stage of lung development.

Altered patterns of cell proliferation in CLD. Immunofluorescence staining for Ki67 and proSP-B in lung sections counterstained with DAPI was performed on the 6d-, 14d-, and 21dPRN animals (Fig. 6). All three PRN groups had apparently increased numbers of cells immunostained for Ki67 (Fig. 6, dashed arrow) particularly when compared with the 140 and 146 GC animals (see Fig. 4, B and C). Lungs from the three PRN groups also had apparently increased cells that were proSP-B positive (Fig. 6, yellow arrows). The 6d- and 14dPRN animals had increased cells that immunostained for both Ki67 and proSP-B (Fig. 6, solid arrows). Some of the proSP-B-positive cells were probably in collapsed distal air spaces. By 21 days of...
Fig. 3. Expression of histone H3 in normal fetal baboon lung and in CLD. Proliferation marker histone H3 was evaluated by in situ hybridization in lung sections from 125 GC (A), 140 GC (B), 6dPRN (C), and 14dPRN (D) animals. Cells expressing histone H3 mRNA decreased between 125 and 140 days gestation. In the PRN animals, an increased number of cells expressed histone H3 mRNA compared with GC animals. Bar, 100 μm.

Fig. 4. Changes in Ki67 and pro-surfactant protein B (proSP-B) in normal fetal baboon lung. Immunofluorescence staining for Ki67 (Texas red) and proSP-B (FITC, solid arrows) was combined with 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent marker for double-stranded DNA (blue nuclei, yellow arrow). Nuclei with Ki67 are magenta (dashed arrows); autofluorescence in red blood cells is orange. During gestation, the number of Ki67-positive nuclei decreased and the number of proSP-B-positive cells increased. Intensity of proSP-B staining in individual cells increased in gestation: 125 GC (A), 140 GC (B), 146 GC (C), and 175 GC (D). Bar, 50 μm.
treatment, the number of Ki67 cells remained high, but these were less likely to be proSP-B positive.

Quantifying these images showed that the proportion of total cells that were proliferating remained elevated in the PRN groups whereas these cells decreased in number in the GC groups (Fig. 7A). In the 14d- and 21dPRN groups, the proportions of Ki67-expressing cells were 2.5- and 8.5-fold greater than in the 140 and 146 GC groups, respectively. The proportions of cells that were immunostained for proSP-B increased more rapidly in the PRN than in the GC treatment, with increased proportions of proSP-B-positive cells in all groups (Fig. 7B). These data suggest that rapid but transient proliferation of proSP-B-positive cells occurred during the first 2 wk in the development of CLD. Similarly, an increased proportion of proSP-B-positive cells (~13%) was Ki67 positive in the 6d- and 14dPRN animals, and this proportion declined for the 21dPRN group (Fig. 8B). Together, these data suggest that CLD was associated with increased cell proliferation that was sustained for at least 21 days. However, the pattern of cell proliferation changed. Proliferation of proSP-B-positive cells occurred during the first 2 wk but declined by the third week when the proportion of proliferating non-proSP-B cells increased.

In fetal baboon lungs, both bronchiolar epithelial cells and terminal air space epithelial cells, probably type II cells, can express SP-B (12). To determine whether type II cells were increased in the distal epithelium, we performed in situ hybridization for SP-C (Fig. 9). In 140 GC animals, scattered cells in the distal air space epithelium expressed SP-C message (Fig. 9A, arrow). In many regions of the 14dPRN lung, however, abundant SP-C-expressing type II cells were noted (Fig. 9B) particularly in distal air spaces close to large airways or blood vessels. These data suggest that some of the proSP-B-expressing cells in the distal epithelium were type II cells.

DISCUSSION

CLD of premature infants results from injury of the developing lung by oxygen, ventilation, and inflammatory mediators. Disordered repair of the injured tissue probably contributes to the pathology of CLD. The outcome is lungs with poor alveolization, an abnormal microvasculature, and fibrosis (10). The pulmonary cellular events associated with CLD are poorly understood, but normal developmental processes such as epithelial cell proliferation and differentiation are probably disrupted by oxidants, mechanical stretch, cytokines, and growth factors. We used a well-characterized model of CLD in extremely premature baboons treated with appropriate oxygen and ventilation to examine cell proliferation and focused on cells in the lung parenchyma. Compared with GC animals, the CLD animals had an increased proportion of proliferating cells and increased proliferation of proSP-B-positive epithelial cells, which likely contributed to an
increased proportion of distal air space epithelial cells that expressed proSP-B. Proliferation of proSP-B-positive cells was notable during the first 2 wk of CLD, but non-proSP-B-positive cell proliferation, mainly interstitial cells, was evident by the third week. These findings suggest that development of CLD is associated with substantially altered patterns of proliferation that may affect multiple developmental events including alveolization, vascularization, and fibrosis.

Because hyperoxia can result in DNA damage and repair (27), we used the nuclear protein Ki67 to mark proliferating cells. Ki67 is not expressed during unscheduled DNA synthesis that occurs during DNA repair (20). Ki67 is required for cells to traverse the cell cycle (32), and its expression correlates well with the mitotic index (4). Unlike proliferating cell nuclear antigen (PCNA), Ki67 has a short half-life, which is an important consideration in developing lungs with a relatively high basal rate of cell proliferation. Some of our findings using Ki67 were confirmed by in situ hybridization for histone H3. Histone H3 also has a short half-life and is not expressed in DNA repair (19, 22). Although Ki67 and histone H3 are highly expressed in the cell cycle, it is possible that cells expressing these markers do not traverse the cell cycle. These markers may identify cells that are blocked at various cell-cycle checkpoints. However, our finding that proSP-B-positive cells accumulate during the first 2 wk of CLD, when these cells have high rates of Ki67 expression, indicates that this marker reflects cell proliferation.

We found that a decreasing proportion of distal lung cells expressed Ki67 as gestation progressed from 125 until 175 days, which suggests a decrease in the overall rate of cell proliferation in the latter stages of gestation. These data are consistent with studies in fetal rats by Adamson and King (3), who used tritiated thymidine to mark DNA synthesis. They noted a substantial decrease in labeled cells, particularly epithelial cells, between 17 and 22 days gestation. Those investigators speculated that the decreased rate of epithelial proliferation corresponded to epithelial differentiation. A declining rate of cell proliferation continues into the postnatal period (37).

Relatively few studies have examined patterns of cell proliferation in chronic lung injury. In adults, several injuries such as those induced by oxidants and silica can damage the alveolar epithelium and endothelium...
Repair of the damaged epithelium during recovery from acute lung injuries is important for reestablishment of normal alveolar architecture (33). Proliferation of type II cells followed by differentiation into type I cells may be a key mechanism for alveolar epithelial repair (2). Chronic fibroproliferative lung disease in humans and experimental animals is also associated with type II cell proliferation (21, 35). Exposure of adult baboons to 60% oxygen for 14 days resulted in type II cell proliferation, which suggests chronic epithelial injury and repair (17). Incomplete repair of severe alveolar injury may result in an epithelium populated by hypertrophic type II cells or cuboidal bronchiolar epithelial-like cells (21, 29, 33). These data suggest a chronic injury-repair cycle in which type II cells proliferate and accumulate but type I cells either die or fail to differentiate from type II cells.

Less is known about cell proliferation in damaged developing lungs, which may have high basal rates of proliferation. In neonatal rats exposed to 85% oxygen for 28 days, Warner et al. (37) found an initial decrease during the first 10 days of exposure followed by a transient increase at 14 days that returned to control values at 28 days. Individual cell populations were not identified. An increase in type II cells was noted in neonatal mice exposed to 100% oxygen for 7 days (7). Very few studies have examined the effects of injury in fetal lungs. Coalson et al. (13) studied baboons delivered at 140 days (75% of term) that were treated with >80% oxygen and ventilation for 21 days. Morphometric analysis indicated
that type II cells were increased in study animals, particularly in areas of hyperexpansion. Increased SP-B and SP-C messages, particularly in peribronchiolar alveoli, were found in baboons delivered at 140 days gestation and treated with 100% oxygen and ventilation (11).

The animals used in the present investigation were very premature (125 days gestation corresponds to 26 wk in the human) and were treated only with sufficient oxygen \[ F_{102} \text{ range, 0.21–0.75} \] and ventilation for 21 days to maintain normal blood gas levels. Despite these appropriate treatment conditions, we found a substantial increase in cell proliferation, particularly distal air space epithelial cells, compared with GC animals. The pattern of cell proliferation was also abnormal. Our findings of increased proSP-B-positive cells, increased immunostaining for proSP-B in distal epithelial cells, and increased SP-C mRNA in CLD are consistent with hypertrophic type II cells repopulating a damaged epithelium. An abnormal alveolar epithelial cell phenotype may influence development of the microvasculature, because type II cells express VEGF, an angiogenic factor that is implicated in lung vascular development and injury (5, 25). In normal postnatal rabbits, proliferating cells did not express VEGF (24). An abnormal distal epithelial cell phenotype may have contributed to decreased VEGF expression and the abnormal microvasculature that we found previously in this premature baboon model (25a).

The mechanisms of distal epithelial cell proliferation in our studies are not known. Modest levels of oxygen stimulate one round of epithelial cell proliferation, whereas \[ F_{102} \text{ levels >0.90 inhibit proliferation} \] (16). Although it is not known how oxygen stimulates proliferation, recent studies (28, 31) revealed that oxygen inhibits proliferation via induction of the cyclin-dependent kinase inhibitor p21Cip1/WAF1/Sdi1. Because p21-dependent growth arrest is unlikely that supplemental oxygen directly stimu-

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Fig. 8. Percentage of proliferating cells that were proSP-B positive and percentage of proSP-B-positive cells that were proliferating. Graphs were constructed as in Fig. 7. A: compared with GC animals (open bars), the 6dPRN and 14dPRN animals (solid bars) had a substantially increased percentage of proliferating cells that were proSP-B positive. By 21 days of treatment, this percentage declined toward control values. B: an increased percentage of proSP-B-positive cells were proliferating in 6dPRN and 14dPRN animals, but this percentage declined at 21 days of treatment. Data are means ± SE; \(* P < 0.05\) compared with corresponding GC animals. Note that the x-axes are not linear, no GCs were available at 131 days, and the scales of the y-axes are different.

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Fig. 9. Increased expression of surfactant protein C (SP-C) in CLD. In situ hybridization for SP-C mRNA in lung sections from 140 GC (A) and 14dPRN (B) animals. GC animals had scattered cells with SP-C message, but the PRN animals had some distal air space sacculles with abundant SP-C-positive cells. Bar, 50 μm.
lated epithelial cell proliferation in the treated baboons. Mechanical strain, which may be associated with mechanical ventilation, can induce lung epithelial cell proliferation (9) and may have contributed to our findings. Similarly, several growth factors such as keratinocyte or hepatocyte growth factors (KGF and HGF, respectively) are mitogenic for type II cells and are expressed in lung injury (1). We were unable to detect KGF by Western analysis in our CLD animals (unpublished observation). Circulating factors such as dihydrotestosterone may stimulate fetal lung epithelial cell proliferation (23). The extracellular matrix (ECM) can have substantial effects on type II cell phenotype and proliferation (8, 36). ECM alterations in baboons with CLD are not known, but other CLDs such as BPD and bleomycin-induced fibrosis may have altered ECMs (10, 35). Dissecting the specific causes of the altered patterns of cell proliferation in this study awaits additional investigation.

This study has several important limitations. For example, the baboons received various levels of inspired oxygen and ventilation to maintain normal blood gas levels. However, the number of animals in each group (3 or 4) is too small to correlate variations in oxygen or ventilator settings with cell proliferation. In addition to oxygen and ventilation, the PRN animals underwent premature delivery and received surfactant, antibiotics, and parenteral and/or enteral nutrition. The effects of these agents on cell proliferation are not known. The GC animals developed normally in uterus and indicated the stage of development and the cell-proliferation pattern that the treated animals would have attained. To prevent selection bias in the quantitative assessment of proliferation, the microscopic fields were chosen randomly using only the DAPI images. Quantification of dual immunofluorescence was performed on one tissue section per animal, and 300–800 cells were analyzed in each of 3–6 fields. Ki67 marks cells in the cell cycle. Because it has a short half-life, we could not identify cells that had just left the cell cycle, and our data may underestimate the rate of proliferation. Although the mechanisms of increased cell proliferation cannot be identified in this model, the premature baboon with CLD is highly reproducible and quantifiable and has several similarities with BPD.

In summary, extremely premature baboons treated with appropriate oxygen and ventilation had substantially altered patterns of cell proliferation compared with gestational controls. For the first 2 wk of life, the treated animals retained a high rate of cell proliferation and proSP-B-positive cell proliferation. During this time, proSP-B-positive cells accumulated in the distal epithelium. By 3 wk of age, the overall rate of cell proliferation remained high, but proliferation of proSP-B-positive cells declined. These alterations in cell proliferation and epithelial cell phenotype may contribute to the pathophysiology of CLD.

The authors thank Jacqueline Coalson, Bradley Yoder, and Vicki Winter for expertise with the premature baboon model and Timothy Stevens for statistical assistance.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-63400 (to W. M. Maniscalco) and HL-58774 (to M. A. O’Reilly).

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