Distinct patterns of apoptosis in the lung during liquid ventilation compared with gas ventilation

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WITH IMPROVEMENTS IN NEONATAL CARE, more preterm infants are surviving and requiring prolonged ventilatory assistance. Conventional mechanical ventilation with supraphysiological concentrations of oxygen (hyperoxia) is often needed to treat critically ill preterm and full-term infants. However, prolonged exposure to hyperoxia and positive pressure during mechanical gas ventilation (GV) is well recognized to cause significant pulmonary injury (18, 25). Emerging evidence indicates that exposure to hyperoxia causes significant apoptosis in lungs of adult and newborn animals ventilated for more than 2 days, and the extent of apoptosis correlates with the physiological and histological abnormalities (19, 21, 26). Liquid ventilation (LV, either total or partial) with perfluorochemical (PFC) liquids, which are inert and have high solubility for respiratory gases, has been proposed as an alternative ventilatory strategy in the management of neonates, children, and adults with severe respiratory failure. LV eliminates alveolar air-liquid interfacial surface tension, improves ventilation-perfusion matching, supports gas exchange at lower inspiratory pressures and reduced inspired oxygen concentrations, and minimizes changes in cardiovascular status (12, 17).

Preterm infants often develop progressive atelectasis and respiratory failure due to surfactant insufficiency and structural immaturity of the lung. LV can potentially be used as an effective treatment for these infants. Wolfson and colleagues (27) reported that the majority of very preterm lambs receiving GV for 3 h had severe respiratory failure and nonhomogeneous lung expansion with proteinaceous luminal debris. In sharp contrast, the majority of animals who received LV had good gas exchange, cardiovascular stability, improved lung expansion, and reduced mortality (27).

To explore the cellular mechanisms associated with the improved physiological profiles in LV animals, we studied 110- and 120-day premature lambs receiving either LV or GV and compared them to unventilated, gestationally age-matched controls. To control for the effects of inspired oxygen tension, we used fraction of inspired O2 (FiO2) = 1 in all ventilated animals. Detailed physiological assessments and histological analyses were used to characterize lung functions and clinical outcomes in these preterm animals. In addition, the extent and pattern of cell death were assessed by in situ terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay, the presence...
of internucleosomal DNA ladders, ultrastructural cell morphology, and the expression of cyclin-dependent kinase (Cdk) 5, an early marker for cells undergoing apoptosis (1, 7, 32).

MATERIALS AND METHODS

Animal preparation. Fifty-six preterm lambs were delivered and studied in two age-matched groups (28 each for 110- and 120-day gestation, term = 147 ± 3 days) and in three arms (8 for controls, 10 for GV, and 10 for LV for each age group) as described previously (27). Briefly, dated-pregnant ewes were sedated, and epidural anesthesia was induced. Each ewe was then restrained, and a cesarean section was performed via a ventral-lateral approach. Fetal lambs were delivered, and sedation and muscle paralysis (pancuronium bromide-0.1 mg/kg) were induced. Unventilated control animals were immediately euthanized, and the lungs were prepared and analyzed as described below. All animals were managed according to the National Institutes of Health regulations and the Guiding Principles in the Care and Use of Animals with the American Physiological Society. In addition, all procedures were approved by the Institutional Animal Care and Use Committee of Temple University.

Ventilation procedures. Forty preterm lambs from 110-day or 120-day gestation stages were randomized to receive either GV or LV LiquiVent (a PFC from Alliance Pharmaceutical, San Diego, CA) as the respiratory medium. In these experimental animals, the jugular vein and carotid artery were cannulated, and a tracheostomy was performed. Arterial blood samples were analyzed. Arterial pH, HCO₃⁻, PaO₂, and PaCO₂ were measured using a blood gas analyzer (Radiometer ABL 330) to determine oxygenation, ventilation, and acid-base status. In addition, the arterial and central venous pressures and heart rates were monitored. The animals were then rehydrated through a series of graded ethanol and water. Sections from each animal were stained with hematoxylin and eosin (H&E) or processed for other analyses as described in Fluorescent TUNEL assay and photomicrography.

Cell culture. Normal human bronchial epithelial cells (NHBE; Clonetics, San Diego, CA) were cultured on six-well Transwell plates (Costar, Corning, NY) coated with 50 mg/ml of rat tail collagen I (Collaborative Biomedical Products, Bedford, MA) in a humidified incubator at 37°C, 5% CO₂. NHBE cells were grown in bronchial epithelial cell growth medium (Clonetics) supplemented with 3 ng/ml bovine serum albumin (Panvera, Madison, WI), 100 μM retinoic acid, and 26 μg/ml bovine pituitary extract (Clonetics). Some cultures were treated with PFC at the apical side for 4 h or with 5 mM H₂O₂ for 4 h to induce apoptosis (6).

Isolation of DNA ladders. Low-molecular-weight DNA fractions were isolated from frozen lung tissues and examined for the presence of internucleosomal DNA ladders as previously described (16, 19). Briefly, 110 mg of lung tissues from ventilated or unventilated lambs were homogenized and centrifugation was performed at 13,000 g to separate the low- and high-molecular-weight fractions of genomic DNA. The supernatant (low-molecular-weight fraction) was digested with ribonuclease and then proteinase K at 37°C for 30 min. DNA was extracted with phenol and phenol-chloroform (1:1), and ethanol precipitated. DNA was resuspended and separated by electrophoresis on 1% agarose gels, visualized by staining with 0.5 μg/ml of ethidium bromide.

Fluorescent TUNEL assay and photomicrography. The in situ TUNEL assay that labels the 3-OH ends of genomic DNA fragments generated by endonucleases during apoptosis was performed as previously described (8, 19). Tissue sections were pretreated by incubation with 30 μg/ml of proteinase K for 15 min at room temperature. The end labeling was per-
formed in the presence of 1 U/ml terminal deoxynucleotidyltransferase, 0.01 mM digoxigenin-11-dUTP (alkali stable), 0.4 mM dATP, and 0.5 mM CoCl$_2$ at 37°C for 1 h. The reaction was terminated by immersing slides in 10 mM EDTA (pH 8.0) for 5 min. After being blocked with 1% BSA in Tris-buffered saline (TBS; 30 min at room temperature), the slides were incubated at room temperature for 1 h in 1:10 dilution of rhodamine-conjugated anti-digoxigenin Fab fragment (Boehringer Mannheim, Indianapolis, IN). After washing the slides in TBS, we used DNA-binding dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) to visualize all the nuclei. Each slide was incubated with 100 µl of 2 µg/ml DAPI (Boehringer Mannheim) at room temperature for 5 min. A G-2A filter was used to visualize rhodamine and the TUNEL-positive cells, and an ultraviolet-2A filter was used to visualize the DAPI fluorescence under an epifluorescence microscope (Nikon, Melville, NY). Photographs were recorded on 35-mm film with a UFX camera system (Nikon) and transferred onto Kodak PhotoCD. The images were digitally adjusted for contrast with Adobe Photoshop 4.0.5 (Adobe Systems, Mountain View, CA). Identical adjustments were made to images from control and experimental slides.

**Electron microscopy analyses.** Tissue samples were deparaffinized from tissue blocks by melting away the paraffin at 68°C in an Isotemp oven (model 630G; Fisher Scientific) for ~1 h, followed by a graded series of rinses with xylene and water. Tissues were then postfixed in 1% osmium tetroxide and dehydrated in ethanol and embedded in LX112 (Ladd, Burlington, VT). Thin sections (60 nm) were cut, stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 10 transmission electron microscope.

**Identification of cell type by immunohistochemistry.** Sections were deparaffinized and hydrated. Endogenous peroxidase activity was quenched with hydrogen peroxide/methanol. Staining with cytokeratin antibodies was performed by the indirect immunoperoxidase method manually or on an automated immunostainer (Ventana Medical Systems, Tucson, AZ) (20). Immunohistochemical analyses were performed as described previously (1). The tissue sections were initially treated with Pro tease I, then 3% H$_2$O$_2$ (to ablate endogenous peroxidase activity), and then incubated with a mixture of anti-cytokeratin antibodies, AE1 and AE3 (Biogenex, San Ramon, CA), or with antibodies against Cdk5 (Santa Cruz Biotechnology, Santa Cruz, CA) (1). Tissue sections were then incubated with biotinylated secondary antibody, peroxidase/streptavidin, and 3-amino-9-ethylcarbazole chromogen (Ventana Medical Systems) for 8 min each. The slides were then counterstained with hematoxylin for 1 min, mounted in aqueous mounting media, and covered with glass coverslips.

**Computer-aided image analysis.** Quantitation of apoptotic cells in ventilated lungs using computer-aided image analysis was carried out as described previously (19). Thirty fields per slide were captured from at least four animals for each group (controls and GV and LV animals) on a Nikon Diaphot microscope equipped with epifluorescence with a xenon light source, a Dage RC300 charge-coupled device camera, and a Shutter Lambda 10 filter wheel with the appropriate filter set (Omega Optical, Brattleboro, VT). The camera and filter wheel interfaced with a Dell computer with Metamorph version 3.5 software (Universal Imaging, West Chester, PA). All fields were captured using identical black and gain levels on both hardware and software settings. The TUNEL-positive nuclei were determined based on relative fluorescent intensity (>190), and only areas >20 pixel were analyzed to exclude any fluorescence anomalies. The average area of a nucleus (determined by averaging 50 nuclei) was used as the standard area. To avoid underestimating the total number of nuclei, we divided the areas of overlapping nuclei by the standard area. The apoptotic index (AI) was calculated as the percentage of TUNEL-positive nuclei divided by the total number of nuclei (visualized with DAPI). For scattergrams, 30 consecutive fields per slide were captured as described above. To quantify the extent of nuclear condensation, cultured lung epithelial NHBE cells were stained with DAPI. The DAPI fluorescence was examined, and images were captured as described above, and the nuclear sizes were analyzed by computer-aided analysis as described previously (6). Data were analyzed for statistical significance using the Student’s t-test and ANOVA, with P < 0.05 considered significant.

**RESULTS**

**LV improves gas exchange and lung morphology compared with GV in premature lambs.** Age-matched preterm lambs (110-day and 120-day gestation groups) were randomly enrolled as unventilated, age-matched controls or into the GV and LV protocol and ventilated for up to 4 h. As expected from previous studies (27), each lamb from the 110-day and 120-day gestation groups survived LV. However, only 50% of the lambs in the 110-day group survived the 4-h GV protocol (mean ventilation duration 2.65 ± 0.4 h), whereas every lamb in the 120-day group survived. Figure 1A shows the measurements of the arterial oxygen tension of the ventilated animals. This figure shows that oxygenation in GV animals deteriorated (arterial oxygen tension <45 Torr), whereas LV animals had consistent, effective oxygenation (arterial oxygen tension >160 Torr). In addition, GV lambs demonstrated significantly (P < 0.05) higher values of carbon dioxide tension (Fig. 1B) and lower values of pH than did the LV animals (Fig. 1C). The vital signs, heart rate, central venous pressure, and mean arterial pressure (MAP) were within normal limits for preterm lambs at this stage of development. Table 1 shows a small but significant difference in MAP between the GV and LV groups independent of age. LV animals, especially the 110-day preterm lambs, demonstrated a greater cardiovascular stability than GV animals throughout the experimental period.

To assess the effects of LV and GV on lung histology, we examined lung sections of the preterm lamb lungs by H&E staining. Figure 2E shows that lungs of unventilated, 120-day preterm lambs were structurally immature with nonhomogenous alveolar expansion and thick-walled gas exchange spaces. Lung sections from GV animals appeared to have cellular hyperplasia with thick alveolar septae, nonhomogeneous expansion, and atelectasis in all the animals studied (Fig. 2, A and C). In contrast, lungs of LV animals showed clear, thin-walled alveoli with relatively uniformed alveolar expansion and minimal atelectasis (Fig. 2, B and D). Numerous aggregates composed of cells with condensed nuclei were observed within the lumina of bronchioles of GV lungs (Fig. 2, A and C), whereas in the LV lungs no such aggregates were observed, and condensed nuclei were sparsely distributed (Fig. 2, B and D).
Apoptosis in ventilated fetal lungs. Nuclear condensation is a morphological hallmark for apoptotic cells (22). To determine if the appearance of the cellular aggregates with the condensed nuclei correlates with the presence of apoptosis in GV lungs, we examined for evidence of apoptosis by assaying for the presence of internucleosomal DNA ladders (16). DNA ladders were readily detected in the homogenates of GV lungs of 120-day lambs (Fig. 3A). Surprisingly, internucleosomal DNA ladders were also detected in LV lung homogenates (Fig. 3A). In contrast, there was no evidence of the DNA ladders in unventilated, control fetal lungs (Fig. 3A). The internucleosomal DNA ladders were also observed in ventilated lungs, but not in the unventilated lungs of 110-day preterm lambs (data not shown). The absence of the internucleosomal DNA ladders in unventilated lungs suggests that apoptosis in the ventilated lungs was not derived from normal programmed cell death during late-stage fetal lung development. Another method of assessing apoptosis is using electron microscopy to reveal the morphological characteristics of apoptotic cells. Figure 3, C and D, shows that airway cells from GV lungs had condensed chromatin, a characteristic of advanced apoptotic cells (Fig. 3C), which was not found in unventilated control lungs (Fig. 3B).

Distinct patterns of apoptosis in GV and LV lungs. Although the presence of DNA ladders and condensed chromatin suggests that apoptosis occurred in the ventilated lungs, the extent of apoptosis and the location or the cell types of the apoptotic cells cannot be easily characterized by these techniques. To determine if the better physiological profile correlates with reduced extent of apoptosis in lungs after LV compared with GV, we performed in situ TUNEL assays on histological sections. Consistent with the results of the DNA ladder assay, TUNEL-positive nuclei were detected in both GV (Fig. 4, A and C) and LV (Fig. 4, B and D) lungs, but rarely in unventilated preterm lamb lungs (Fig. 6B). Comparison of the TUNEL-positive signals in GV with LV lungs (Fig. 4) indicates that the two ventilatory modes resulted in distinctly different patterns of distribution of the TUNEL-positive cells. The TUNEL-positive cells in GV lungs were predominantly located at levels of bronchiole, either at the epithelial lining or clustered together in aggregates within the lumina (Fig. 4, A and C). The location of the condensed nuclei observed from the H&E staining (Fig. 2C) is similar to the distribution of TUNEL-positive cells, and both signals were focal and prominent in the more proximal airways of GV lungs. In addition, very few cells at the more distal air exchange spaces were TUNEL positive in these GV lungs. In contrast, TUNEL-positive cells were diffusely distributed throughout the parenchyma.

Table 1. Measurement of MAP, CVP, and HR

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<th>110 Days</th>
<th>120 Days</th>
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<tr>
<td></td>
<td>GV</td>
<td>LV</td>
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<tr>
<td>HR, beats/min</td>
<td>183 ± 7.3</td>
<td>191 ± 3.3</td>
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<tr>
<td>CVP, mmHg</td>
<td>9 ± 0.72</td>
<td>6 ± 0.6</td>
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<tr>
<td>MAP, mmHg</td>
<td>53 ± 2.6</td>
<td>43 ± 2.2*</td>
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Values are means ± SE (n = 10). Preterm lambs at gestational ages of 110 and 120 days were used. MAP, mean arterial pressure; CVP, central venous pressure; HR, heart rate; GV, gas ventilated; LV, liquid ventilated. *P < 0.05 GV vs. LV.
of LV lungs. No clusters of TUNEL-positive cells were found at the more proximal small airways of LV lungs as in GV lungs, although TUNEL-positive cells could be clearly observed throughout the lung including alveoli and interstitial spaces (Fig. 4, B and D).

To further confirm that apoptosis had occurred in ventilated lungs, we examined the expression of Cdk5, a characteristic of the early phase of apoptosis (1, 7, 32). Figure 5, A–C, shows that GV induced Cdk5 expression at the lining and within the lumina of bronchioles. In contrast, Cdk5-positive signals in LV lungs were more evenly distributed throughout the lung parenchyma (Fig. 5D). Similar to the TUNEL-positive signals (Fig. 6B), no Cdk5 expression was detected in the unventilated lungs (data not shown). The localization of the Cdk5-positive signals in ventilated lungs (Fig. 5) is similar to the distribution of TUNEL-positive signals (Fig. 4), suggesting that LV and GV induce apoptosis in the preterm lungs, but with distinctly different distribution patterns.

To quantitatively examine the differences in the distribution of apoptotic cells, we subjected TUNEL-positive fluorescent images to computer-aided image analyses. Thirty adjacent fields from tissue sections of at least four GV and LV animals each were analyzed. Panels G and H in Fig. 4 are scattergrams of the TUNEL-positive signals in each field of view. Figure 4G is a representative lung section illustrating the focal nature of the TUNEL-positive signals in GV lungs, where a single field had 95 TUNEL-positive nuclei, whereas the majority of the adjacent fields had less than five TUNEL-positive nuclei. In contrast, TUNEL-positive nuclei from LV animals were more evenly distributed in each field of view throughout the entire lung (Fig. 4H). To measure the overall extent of apoptosis in ventilated lungs, we determined the AI, defined as the percentage of TUNEL-positive nuclei in each field divided by the total number of nuclei, assessed by DAPI staining (19). Analysis of AI indicated that both LV and GV induced marked apoptosis. However, the AI in animals receiving LV (9.9% ± 1.7%) is significantly higher than those receiving GV (3.3% ± 0.9%), whereas the AI in the unventilated animals was significantly lower (0.77% ± 0.08%) than either ventilation group.

Apoptosis of epithelial cells in GV lungs. In adherent cell cultures, apoptotic cells are often detached from the culturing surface (16). To determine whether the
apoptotic cells within the lumina of the bronchioles in GV animals are epithelial cells, we stained lung sections with antibodies against cytokeratin, an epithelial cell-specific marker. Figure 6A shows that cells at the lining of small airways in unventilated lungs were epithelial in origin and were TUNEL negative (Fig. 6B). In GV lungs, the majority of cells located at the lining and within the lumina of bronchioles were cytokeratin positive (Fig. 6, C and E), whereas some of them were also TUNEL positive (Fig. 6, D and F). In addition, some of the TUNEL-positive epithelial cells within the lumina of bronchioles seemed connected with epithelial cells at the lining of bronchioles (Fig. 6, E and F). This notion that the TUNEL-positive cells within the lumina of the bronchiole in GV lungs are epithelial was further supported by results shown in Fig. 6, G and H (from adjacent lung sections), which shows that aggregated TUNEL-positive cells also stained positive for cytokeratin. At the peripheral/lumina of some of the more proximal airways in GV lungs, the percentage of TUNEL-positive epithelial cells was as high as 60–70%. However, this high percentage of TUNEL-positive epithelial cells clustered only at the more proximal airways, suggesting a focal nature of lung apoptosis in GV animals.

**PFC does not induce apoptosis of cultured lung epithelial cells.** To examine whether PFC alone can induce apoptosis of lung epithelial cells, we cultured NHBE cells at the air-liquid interface and exposed them to PFC for 4 h. Apoptosis was assessed by nuclear condensation as described previously (6). No significant apoptosis was observed in NHBE cells exposed to PFC for 4 h at the apical side (total nuclear area was $1,338 \pm 79$ pixels), whereas cells exposed to $H_2O_2$ had most condensed nuclei (total nuclear area was $381 \pm 16$ pixels). Cells cultured at room air without exposure to either PFC or $H_2O_2$ were used as controls for normal nuclear morphology (total nuclear area was $1,470 \pm 192$ pixels).

**DISCUSSION**

In this report, we demonstrate that preterm lambs (55 or 65% of term gestation) ventilated with LV had improved pulmonary function and gas exchange with better lung inflation patterns compared with GV and
Fig. 4. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assays of GV and LV lungs. A–D: fluorescent TUNEL assays were performed on paraffin sections of lungs of lambs of 120-day gestation ventilated for 4 h with either GV (A, C) or LV (B, D). E and F: the corresponding nuclei of C and D, respectively, stained with 4–6-diamidine-2-phenylindole-dihydrochloride. G and H: scattergrams illustrating the number of TUNEL-positive nuclei per field in 30 consecutive fields in lungs from 120-day gestation fetal lambs ventilated with either GV (G) or LV (H) for 4 h. Scale bars: A and B, 50 μm; C–F, 20 μm.
unventilated animals. In contrast, animals receiving GV suffered from high mortality with poor gas exchange, atelectasis, and hypoxemia despite ventilation with 100% O₂. In addition, both LV and GV caused marked apoptosis in the lungs of preterm lambs. More importantly, the distribution patterns of the apoptotic cells are distinctly different between these two modes of ventilation. Preterm lambs exposed to GV had clusters of apoptotic cells in the lining and within the lumina of more proximal airways. In contrast, LV lungs had a more diffuse and homogeneous pattern of apoptosis throughout, including central airways and gas-exchange areas.

Prolonged exposure to conventional gas ventilation with hyperoxia causes acute inflammatory changes in the lung that may then evolve into acute lung injury and chronic lung disease (e.g., bronchopulmonary dysplasia) (24). The resulting severe respiratory failure in preterm infants is associated with significant morbidity and mortality. LV has been proposed as an alternative ventilatory strategy for the treatment of these infants. Since 1989, a number of clinical trials using LV have been performed in infants with severe respiratory distress syndrome unresponsive to surfactant and conventional ventilation treatment (11). Overall, LV appears to improve lung function of the treated infants without adverse effects. In lamb models with severe respiratory failure, LV has been shown not only to improve oxygenation and maintain gas exchange, but also to minimize lung injury (less hemorrhage, edema, and inflammation) compared with conventional GV (12, 17).

In preterm lambs, surfactant insufficiency and structural immaturity of the lung result in abnormal alveolar surface tension and marked reductions in lung compliance. The present studies indicate that conventional ventilation with hyperoxia cannot overcome these intrinsic problems in the lung of these preterm lambs. As shown previously (27), the preterm animals receiving GV suffered high mortality and morbidity with gas exchange failure, ineffective CO₂ elimination, and inability to maintain acid-base balance (Fig. 1). Despite ventilation with 100% O₂, GV animals were still hypoxemic (Fig. 1). The primary cause of the gas exchange abnormalities appears to be atelectasis. One simple explanation for the atelectasis in GV animals is that the immature alveolar surface characteristics of the preterm animals result in lethal gas exchange failure due to the intrapulmonary shunt. In contrast, PFC minimizes interfacial surface tension forces in the immature lungs and lessens atelectasis by abolishing the effects of an air-liquid interface throughout the lung. As shown in Fig. 2, the gas exchange areas in LV lungs were more homogeneously expanded compared with both GV and unventilated lungs. In addition, LV optimized CO₂ elimination and acid-base balance (Fig. 1), further improving pulmonary functions in these animals. Together, these results further support previously published studies indicating that LV significantly improves clinical respiratory status and lung histology in preterm lambs.

Reports in the literature have suggested that the TUNEL assay detects either necrotic or apoptotic nuclei (10). To clarify the TUNEL results that suggest...
cells undergo apoptosis in ventilated preterm lungs, we further examined the expression of Cdk5, a marker for early apoptosis. Cdk5 is known to be particularly important in the regulation of apoptosis during early development (1, 7, 32). In addition, TUNEL-positive signals and Cdk5-positive cells have been shown to be colocalized in injured lungs during bacterial pneumonia (14). Figures 4 and 5 show that the patterns of Cdk5 expression in both GV and LV lungs were similar to the ones of TUNEL-positive signals, suggesting that...
TUNEL-positive signals correlate with the expression of this apoptotic gene in ventilated preterm animals. The morphological characteristics (Fig. 3C) and the presence of internucleosomal DNA ladders (Fig. 3A) further support the occurrence of apoptosis in these ventilated lungs. Together, these results demonstrate that cells, including epithelial cells, undergo apoptosis in ventilated preterm lamb lungs.

Apoptosis is known to play an important role in the development of many tissues and internal organs, including the digits (29, 30) and early embryonic lungs (3, 15, 23). In the development of human lungs, apoptosis is evident in fetal lungs up to 24 wk of gestation (23). Fetal lambs of 110 and 120 days are equivalent to ~22 and 26 wk of gestation in infants, respectively, with respect to lung development. No marked apoptosis was observed in these fetal lungs (Figs. 3 and 6). This observation suggests that apoptosis is not a prominent feature in stages of fetal lung development studied herein and cannot account for the marked apoptosis in ventilated preterm lambs.

Exposure to hyperoxia may be important in the pathogenesis of lung apoptosis in ventilated preterm lambs. Results from apoptotic assays including DNA ladders, TUNEL, and Cdk5 expression indicate that apoptosis is a prominent phenomenon in GV as well as LV lungs (Figs. 3–6). Because of the surfactant insufficiency and structural immaturity in the lung, it is technically challenging to sustain the life of these preterm lambs for 4 h without ventilating them with hyperoxia. Therefore, it is difficult to perform experiments that examine the effects of PFC alone on lung cell apoptosis in preterm lambs. However, several lines of evidence suggest that there is a relationship between exposure to hyperoxia and lung apoptosis. First, studies in several adult or newborn animal models have demonstrated that apoptosis occurs in the lungs of animals exposed to conventional ventilation with 100% oxygen (2, 19, 21, 26). The extent of apoptosis also correlates with the severity of lung injury in these systems, suggesting that apoptotic cell death is important in the pathogenesis of hyperoxic lung injury (19, 21, 26). Second, direct exposure of cultured primary and transformed lung epithelial cells to hyperoxia causes cell injury and cell death (13). Third, direct exposure of cultured lung epithelial cells to PFC alone neither induces apoptosis nor inhibits proliferation of these cells (unpublished results), suggesting that apoptosis in LV lungs does not result from toxic effects of PFC on lung epithelial cells. Fourth, the distribution of apoptotic cells in both GV and LV lungs correlates with regions of the lung exposed to ventilation with hyperoxia. LV animals had well-expanded, oxygenated lungs (Figs. 1 and 2), and a homogeneous pattern of apoptosis was observed throughout the lungs, including the airways, gas exchange areas, and the interstitial spaces (Figs. 4 and 5). In contrast, GV animals had profound atelectasis. The gas exchange areas of the lung were not exposed to hyperoxia (Figs. 1 and 2), and apoptotic cells were rare in these regions (Figs. 4–6). Finally, cells in the more proximal central airways of both LV and GV lungs are exposed to hyperoxic conditions because both groups were ventilated with high inspired oxygen concentrations, and apoptosis was detected in central airway regions of both GV and LV animals. Although GV animals had a significantly higher extent of apoptosis (as high as 60–70%) in the lining and within the lumina of bronchioles, LV lungs had a more homogeneous AI (9.9 ± 1.7%) than the rest of the lung (Figs. 4 and 6). The epithelial origin of the apoptotic cells within the lumina of airways of GV lungs (Fig. 6) suggests that these cells were sloughed off from the epithelial lining of the airways. The apoptotic epithelial aggregates in the lumina of these airways may result from hyperoxia-induced cell injury and death of both epithelial and phagocytic cells that were unable to be cleared. In contrast, no clusters of apoptotic cells were observed in the airway lumina of LV lungs. The steady-state levels of apoptosis at any given time are determined not only by how many cells undergo apoptosis, but also by how quickly apoptotic cells are cleared. One simple explanation for the low extent of apoptosis in the proximal airways of LV lungs is that the continuous flow of PFC through the lungs may facilitate the clearance of the detached, apoptotic cells. Together, these studies suggest that there is a link between apoptosis and the exposure to hyperoxia in ventilated preterm animals. The higher extent of apoptosis in LV compared with GV animals is most likely related to the enhanced distribution of the inspired oxygen tension. Although GV lungs remained atelectatic, precluding exposure of the alveolar-capillary membrane to high levels of oxygen, improved recruitment with LV fostered a better distribution of the inspired oxygen, higher arterial oxygenation, and more exposed lungs to the high inspired oxygen tension compared with GV. Therefore, LV provides an opportunity to titrate the inspired oxygen tension to reduce the risk for apoptosis compared with GV. Future studies are necessary to determine the optimal FiO2/PFC combination that will increase survival while minimizing injury in the preterm animals.

The exact role of apoptosis in the pathogenesis of preterm lung injury is not clear. The overall AI throughout GV or LV lungs does not correlate with the clinical outcomes of these preterm animals, suggesting that the relationship between the clinical respiratory status and the extent of apoptosis is not a simple correlation, especially when the comparison is made in lungs ventilated with different media. However, as discussed above, the distribution and localization of apoptotic cells in these preterm lungs may better explain this discrepancy. Apoptosis in ventilated preterm lambs may be an indicator for cell injury and DNA damage associated with hyperoxic lung injury as shown in adult animals (2, 19, 21, 26). The apoptotic aggregates in the lumina of the more proximal airways of GV lungs may have some impact on atelectasis and hypoxemia in the gas exchange areas, observed in all GV animals studied. However, in a complex organ such as the lung, the temporal apoptotic pattern and cell types involved are necessary to define the role and
mechanism of apoptosis. More importantly, in preterm animals, it is unclear whether apoptosis in LV lungs is an indicator for remodeling/maturation of preterm lungs besides being a simple marker for cells exposed to hyperoxia.

In summary, the present studies indicate that atel-ectasis plays a major role in the loss of pulmonary function, cardiovascular stability, and gas exchange, which leads to arterial hypoxemia and increased mortality in preterm lambs receiving GV. LV provides a more optimal ventilatory strategy with improvements in oxygenation, pulmonary function, and lung inflation patterns, although evidence of lung injury was still seen. Although both modes of ventilation induce lung apoptosis in preterm animals, the patterns of apoptosis are distinctly different, with a correlation between apoptosis and the exposure to hyperoxia. While the role of apoptosis in lung development is yet to be defined, our studies suggest that apoptosis in both airway and lung parenchyma may be minimized by LV with lower inspired oxygen tension.

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