Persistent bronchiolar remodeling following brief ventilation of the very immature ovine lung

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O’Reilly M, Hooper SB, Allison BJ, Flecknoe SJ, Snibson K, Harding R, Sozo F. Persistent bronchiolar remodeling following brief ventilation of the very immature ovine lung. Am J Physiol Lung Cell Mol Physiol 297: L992–L1001, 2009. First published August 28, 2009; doi:10.1152/ajplung.00099.2009.—Children and adults who were mechanically ventilated following preterm birth are at an increased risk of reduced lung function, suggesting small airway dysfunction. We hypothesized that short periods of mechanical ventilation of very immature lungs can induce persistent bronchiolar remodeling that may adversely affect later lung function. Our objectives were to characterize the effects of brief, positive-pressure ventilation per se on the small airways in very immature, surfactant-deficient lungs and to determine whether the effects persist after the cessation of ventilation. Fetal sheep (0.75 of term) were mechanically ventilated in utero with room air (peak inspiratory pressure 4 cmH2O, positive end-expiratory pressure 4 cmH2O, 65 breaths/min) for 6 or 12 h, after which the small airways in very immature lungs can induce persistent bronchiolar remodeling that may adversely affect later lung function. We characterized the effects of brief, positive-pressure ventilation per se on the small airways in very immature, surfac-
tant-deficient lungs and to determine whether the effects persist after the cessation of ventilation. Fetal sheep (0.75 of term) were mechanically ventilated in utero with room air (peak inspiratory pressure 4 cmH2O, positive end-expiratory pressure 4 cmH2O, 65 breaths/min) for 6 or 12 h, after which tissues were collected; another group was studied 7 days after 12-h ventilation. Age-matched unventilated fetuses were controls. The mean basement membrane perimeter of airways analyzed was 548.6 ± 8.5 μm and was not different between groups. Immediately after ventilation, 21% of airways had epithelial injury; in airways with intact epithelium, there was more airway smooth muscle (ASM) and less collagen, and the epithelium contained more mucin-containing and apoptotic cells and fewer proliferating cells. Seven days after ventilation, epithelial injury was absent but the epithelium was thicker, with greater cell turnover; there were increased amounts of bronchiolar collagen and ASM and fewer alveolar attachments. The increase in ASM was likely due to cellular hypertrophy rather than hyperplasia. We conclude that brief mechanical ventilation of the very immature lung induces remodeling of the bronchiolar epithelium and walls that lasts for at least 7 days; such changes could contribute to later airway dysfunction.

epithelium; airway smooth muscle; collagen; proliferation; apoptosis

ADVANCES IN PERINATAL CARE have led to improved survival following very preterm birth, with infants born as early as 23–24 wk of gestation now being capable of survival and leading essentially normal lives. However, numerous studies investigating the long-term outcomes of very preterm birth have shown that children (14, 17, 25, 27) and young adults (15) who were born preterm (<32 wk of gestation) are at an increased risk of having impaired lung function, including reductions in forced expiratory volume in 1 s (FEV1) and forced expiratory flow rates (FEF25–75%). Together, these follow-up studies suggest that the small conducting airways may be permanently affected by very preterm birth, especially if these infants subsequently developed bronchopulmonary dysplasia (BPD).

Histological studies of the lungs of infants who were mechanically ventilated following preterm birth have shown evidence of damage to the large and small airways. In the larger airways, a loss of epithelium from the trachea and main bronchi (28) and an increased area of submucosal glands (18, 30) have been reported. Studies that have investigated the smaller airways have shown a greater amount of airway smooth muscle (ASM) (18, 19, 39, 43), increased numbers of goblet cells and altered epithelial cells (18, 43). Similar findings have been observed in animal models of ventilator-induced lung injury following preterm birth. In preterm ventilated baboons, the small airways had hyperplastic changes in the epithelium (9), and in preterm ventilated lambs, the terminal bronchioles had increased amounts of ASM (1). Although it is generally considered that these alterations in airway wall structure contribute to the reduced lung function experienced later in life, the etiology of structural remodeling of the conducting airways and its persistence remain incompletely understood.

Infants born very preterm often require mechanical ventilation because their lungs are structurally immature and surfactant deficient. Mechanical ventilation is thought to contribute to the develop-
ment of BPD and chronic lung disease, although numerous other factors associated with preterm birth could be causally involved, including exposure to hyperoxic gas, infections, or poor nutrition (2). Because of these potentially causal factors, the effects of mechanical ventilation per se on the immature airways are poorly understood, and there is now a recognized need to separate their independent effects (38). Our hypothesis was that even short periods of positive-pressure mechanical ventilation of the very immature lung, in the absence of other physiological factors, can cause persistent changes in the wall structure of the small conducting airways. Although it has long been known that short periods of mechanical ventilation can acutely injure the preterm tracheal (11) and bronchiolar epithelium (31, 33), little is known about the effects of ventilating the very immature lung on other components of the bronchiolar walls or the persistence of such changes.

The interpretation of previous studies of the effects of ventilation on the developing airways is rendered difficult by the inability to isolate individual injurious factors associated with maintaining a prematurely delivered animal or infant. To avoid potentially confounding factors associated with maintaining life following very preterm birth, together with the importance of ventilating the lung at a stage of lung development that is representative of very preterm infants (i.e., those
most likely to develop BPD and later respiratory complications), we chose to ventilate the lungs of fetal sheep in utero. Recently, we reported that in utero ventilation provides a means by which individual factors involved in the development of BPD can be studied, since the subject is supported metabolically by the mother and is in an aseptic environment (3). At the gestational age of ventilation (0.75 of term), the ovine fetal lung is in the canalicular (i.e., prealveolar, presurfactant) stage of development and therefore is comparable to the lungs of very preterm infants. Our objectives were to determine 1) the effects of short periods of mechanical ventilation per se on the wall structure of small conducting airways (i.e., bronchioles) of the very immature lung and 2) the persistence of any induced structural changes following the cessation of ventilation.

**MATERIALS AND METHODS**

**Fetal Surgery and Treatment Groups**

Experimental procedures were approved by the Monash University Animal Ethics Committee (3). Under general anesthesia (1.5% halothane in O₂), the fetuses of time-mated pregnant ewes (Border Leicester × Merino) underwent aseptic surgery at 105 days of gestational age (DGA); full term in this breed of sheep is ~147 DGA. During surgery, an endotracheal tube was inserted into an incision in the midcervical fetal trachea, directed toward the lungs, and connected to two saline-filled tubes (inner diameter 9.5 mm, outer diameter 14.3 mm) via a Y-piece (3). A saline-filled catheter (inner diameter 3.2 mm, outer diameter 6.4 mm) was inserted into the ascending fetal trachea and connected (external to the ewe) to one of the saline-filled ventilation tubes. This created an exteriorized tracheal loop, which allowed the normal flow of fetal lung liquid during nonventilation periods (3). After the surgical period and throughout the experimental period, ewes were housed in individual cages with no further anesthesia.

The animals were randomly divided into five treatment groups (n = 5 fetuses/group). The immediate effects of ventilation were studied in three groups of fetuses at 110 DGA: the first group was ventilated for 6 h, followed by 6 h of recovery during which lung liquid was replaced (6h+6h group); a second group was ventilated for 12 h (12h group); and a third group comprised sham-operated, age-matched control fetuses (110 DGA controls). These three groups of fetuses were killed immediately after the 12-h study period at 110 DGA. We chose to ventilate the animals for 6 and 12 h so that we could assess the effects of duration of ventilation on airway injury. To determine whether the effects of ventilation persisted for 7 days, we used two additional groups of fetuses killed at 117 DGA: one group was ventilated for 12 h at 110 DGA, lung liquid was then replaced, and the fetus was allowed a 7-day recovery period in the uterus with no further ventilation (12h+7d group). The second group served as age-matched control fetuses (117 DGA controls).

Sham-operated control fetuses had the same surgical procedures performed at the same gestational age as the ventilated fetuses (3). On the day of experimentation, sham-operated fetuses again received identical treatment with the exception of ventilation. In all fetuses, tracheal pressure was monitored after surgery and experimentation (or sham experimentation); there was no evidence that the insertion of the tracheal catheters affected tracheal fluid pressure or flow.

**Ventilation Procedure**

Details of the in utero ventilation procedure have been reported previously (3). Before the commencement of ventilation, fetal lung liquid was drained via the descending tracheal tube and stored; this tracheal tube was then connected to a ventilator (Draeger 8000+). Fetuses underwent positive-pressure ventilation with unhumidified room air at 65 breaths/min with a peak inspiratory pressure of 40 cmH₂O, a peak end-expiratory pressure of 4 cmH₂O, and an inspiratory flow of 15 l/min. These ventilatory parameters and the use of unhumidified air were chosen to cause injury to the immature lungs, to replicate the lung injury seen in human BPD. Because our flow sensor for the measurement of tidal volume would not function in utero, we assessed tidal volumes using the same ventilatory parameters in a separate group of anesthetized sheep (n = 3) while the fetal head was exteriorized but the rest of the fetus remained in utero. In these fetuses, the tidal volume was 3–5 ml/kg during the ventilation period (3). Since these tidal volumes are comparable to those used in preterm infants, we do not consider that volutrauma or excessive shear force occurred during the ventilation period.

**Tissue Collection and Preparation**

At the end of the study periods, ewes and fetuses were humanely killed by an overdose of pentobarbital sodium administered to the ewe (130 mg/kg iv). Neither the control nor the treated fetuses appeared to have overt inflammation surrounding the wound sites (ewe’s flank or the fetal trachea) when observed at necropsy. The fetal lungs were removed and weighed, after which the left bronchus was ligated and the left lung removed distal to the ligature. The right lung was fixed via the trachea at 20 cmH₂O (4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4), post-fixed in Zamboni’s fixative overnight, and processed for light microscopy. Paraffin-embedded sections of lung tissue (5 μm thick) were stained with either Masson’s Trichrome stain (general morphology), Gordon and Sweet’s reticular fiber stain (collagen), or the periodic acid-Schiff’s stain (PAS; mucus). Immunohistochemistry was performed on sections to localize α-smooth muscle actin (α-SMA; as a marker of ASM), Ki67 (to measure cell proliferation), and active caspase-3 (to measure cell apoptosis) using specific antibodies as described below.

**Airway Wall Analysis**

Stained sections were examined using a light microscope, and color images were captured using a digital camera linked to image analysis software (Image Pro Plus, version 6.0). The sections of lung tissue were scanned at a magnification of ×400, and all noncartilaginous airways that appeared in a near-circular cross section were included in the analysis; the approximate diameter of the airways analyzed ranged from 70 to 320 μm. The airways analyzed were all noncartilaginous as small as terminal bronchioles; their generation number could not be determined because these airways are too small to be dissected out in the way that larger airways can be. Airways were categorized as intact if their epithelium was continuous or injured if the epithelium was detached or absent. For each morphometric parameter, we obtained data from a total of 15 randomly chosen airways in 5 randomly selected sections (taken from the 3 blocks per lobe that were collected) from each animal; these included at least 1 section from the upper, middle, and lower lobes of the lung. All analyses were performed on coded slides by a single observer (M. O’Reilly) who was blinded to the experimental groups.

**Morphometric Analysis of Intact Airways**

Using light microscopy, we measured the luminal area, the perimeter of the basement membrane (P₂m), the area of airway epithelium, the number of proliferating, apoptotic, and mucus-containing cells in the airway epithelium, the area of ASM, the area of collagen in the outer airway wall, and the number of alveolar attachments.

**Perimeter of the basement membrane.** P₂m was used as an index of airway size, and measurements of airway wall components were normalized by dividing by P₂m. P₂m has been shown to be a suitable marker of airway dimension because it does not change significantly with changes in lung volume or bronchoconstriction (4). This normalization is necessary to correct for the fact that the bronchioles analyzed may have been from different generations of airways.
**Area of epithelium.** The area of the epithelium was calculated as the difference between the area enclosed by the epithelial basement membrane and the area of the lumen.

**Cell proliferation and apoptosis.** Sections of lung tissue were incubated with a specific antibody against Ki67 (anti-Ki67, 1:100; M7240, DakoCytomation) or active caspase-3 (anti-caspase-3, 1:1,000; AF835, R&D Systems) using an immunohistochemistry kit [EnVision + Dual Link System-HRP (DAB+), DakoCytomation]; sections were counterstained with hematoxylin to identify epithelial cell nuclei. The numbers of proliferating (Ki67 positive) and apoptotic (active caspase-3 positive) cells in the epithelium were counted and expressed as a percentage of the total number of epithelial cell nuclei counted. ASM cell proliferation and apoptosis were determined by counting Ki67- and caspase-3-positive cells, respectively, in the ASM and expressing the number of airways that contained proliferating or apoptotic ASM cells as a percentage of the total number of airways analyzed.

**Mucin-containing cells.** Sections stained with PAS were used to identify mucin-containing cells in the airway epithelium; PAS is a marker for mucus glycoproteins secreted by goblet cells. The number of mucin-containing cells was expressed as a percentage of the total number of epithelial cells counted.

**Area of ASM.** To assess the area of ASM, we incubated lung tissue sections with a specific antibody against α-SMA (anti-α-SMA, 1:500; M0851, DakoCytomation) or active caspase-3 (anti-caspase-3, 1:1,000; AF835, R&D Systems) using an immunohistochemistry kit [EnVision + Dual Link System-HRP (DAB+)]; sections were counterstained with hematoxylin. The ASM area in the outer airway wall was expressed relative to P_bm.

**Area of airway collagen.** Sections stained with Gordon and Sweet’s reticular fiber stain were used in the measurement of type I and type III collagen in the outer airway wall. The area of collagen was expressed relative to P_bm.

**Alveolar attachments.** These are the points where alveolar walls are radially attached to the outer wall of the airways (36); they were quantified as the number of attachment points around the margin of bronchioles in relation to the P_bm.

**Qualitative Analysis of Injured Airways**

Injured airways were those with a detached or absent epithelium (Fig. 1A). Because the walls of the injured airways could not be morphometrically analyzed using the same criteria as the intact airways, the damage was assessed qualitatively. The proportion of airways that were injured in each treatment group was determined by expressing the number of injured airways as a percentage of the total number of airways (intact and injured) in all tissue sections analyzed. The severity of epithelial damage was scored as mild, moderate, or severe. Mild damage was defined as <45 degrees of epithelium being detached or lost, moderate damage if 45–180 degrees of epithelium was detached or lost, and severe if >180 degrees of epithelium was detached or lost; this scoring technique was modified from methods described in previous studies (26, 32).

**Data Analysis**

Results are means ± SE. Comparisons between treatment groups were made using a nested analysis of variance (ANOVA), with number of airways, lung lobe, and treatment as factors. After a significant nested ANOVA, a least significance difference post hoc test was conducted. A P value <0.05 was taken as indicating that values were significantly different.

**RESULTS**

**Fetal Homeostasis**

Fetal arterial P_{O_2}, P_{CO_2}, pH, and SO_2 were measured before and during the ventilation procedure, during which the fetuses were maintained on placental support (3). All fetuses had normal arterial gas tensions and pH throughout the experiments, and there were no significant differences between ventilated and control fetuses (data not shown). In ventilated fetuses, arterial gas tensions and pH during the period of ventilation did not differ from pre-ventilation values. At necropsy there were no significant differences between the ventilated and control fetuses in body weight or weights of the lungs, heart, liver, and kidneys.

**Airway Size**

The mean P_{b_m} of the airways analyzed was 548.6 ± 8.5 μm, and values ranged from 210 to 1,015 μm; the majority (~80%) of airways analyzed had P_{b_m} values between 300 and 700 μm (Fig. 2A). There was no significant difference in mean P_{b_m} among the five study groups: it was 548.0 ± 17.2 μm in the 6h+6h group, 536.1 ± 23.6 μm in the 12h group, 563.5 ± 19.8 μm in the 110 DGA controls, 527.2 ± 17.6 μm in the 12h+7d group, and 563.8 ± 18.7 μm in the 117 DGA controls. The frequency distribution of P_{b_m} values of the airways analyzed was similar in each study group (Fig. 2, B–F).

**Proportion of Intact and Injured Airways**

The proportion of injured airways, expressed relative to the total number of airways in all tissue sections analyzed, was significantly greater (P < 0.001) in the 12h group (29.7 ± 10.7%) than in 110 DGA controls (6 ± 0.5%; Fig. 1B and Table 1). Injured airways were found in all fetuses in the 12h group. In 6h+6h fetuses, the percentage of damaged airways was 10.2 ± 4.2%; however, this was not significantly different from that in age-matched (110 DGA) controls. There was no difference in the incidence of injured airways between 12h+7d fetuses (0.8 ± 0.4%) and age-matched (117 DGA) controls (0.5 ± 0.3%).

**Morphometric Analysis of Intact Airways**

**Airway epithelium.** The epithelial area, relative to P_{b_m}, in the 6h+6h group (11.6 ± 0.4 μm^2/μm) and 12h group (11.1 ± 0.3 μm^2/μm) was not significantly different from values in 110 DGA controls (11.2 ± 0.2 μm^2/μm; Fig. 3). The epithelial area in 12h+7d fetuses (10.0 ± 0.2 μm^2/μm) was significantly greater (P < 0.001) than in 117 DGA controls (8.2 ± 0.2 μm^2/μm). In control animals, the epithelial area at 117 DGA was significantly smaller than at 110 DGA.

**Proliferating cells in the airway epithelium.** The percentage of epithelial cells undergoing proliferation (Fig. 4A) in 12-h fetuses (2.8 ± 0.8%) was significantly lower (P < 0.05) than in 110 DGA controls (5.5 ± 0.8%); values in 6h+6h fetuses (5.2 ± 0.8%) were not different from controls. Fetuses in the 12h+7d group had a significantly higher percentage of proliferating epithelial cells (3.6 ± 0.5%, P < 0.001) than in 117 DGA controls (0.2 ± 0.1%). In control animals, the percentage of proliferating epithelial cells was significantly greater (P < 0.001) at 110 DGA than at 117 DGA.

**Apoptotic cells in the airway epithelium.** The percentage of epithelial cells undergoing apoptosis (Fig. 4B) in 12h fetuses tended to be higher than in 110 DGA controls (1.0 ± 0.4 vs. 0.4 ± 0.1%, P = 0.059) and was not significantly different in 6h+6h fetuses (0.4 ± 0.1%). The percentage of apoptotic epithelial cells in 12h+7d fetuses was significantly greater than...
in 117 DGA controls (0.7 ± 0.2 vs. 0.2 ± 0.1%, \( P < 0.05 \)). In control animals, the proportion of epithelial cells undergoing apoptosis was similar at 110 and 117 DGA.

Mucin-containing cells in the airway epithelium. The percentage of mucin-containing cells in the airway epithelium (Fig. 4C) of 12h fetuses (41.0 ± 3.2%) was significantly greater than in 110 DGA controls (25.5 ± 2.9%). There was no significant difference in the proportion of mucin-containing cells between the 6h+6h fetuses (23.0 ± 3.1%) and their 110 DGA controls or between 12h+7d fetuses (46.2 ± 3.2%) and their 117 DGA controls (54.6 ± 4.3%). In control animals, the percentage of mucin-containing cells at 117 DGA was greater than at 110 DGA (\( P < 0.001 \)).

Airway smooth muscle. The area of ASM (Fig. 5A), relative to \( P_{bm} \), was significantly greater (\( P < 0.05 \)) in 6h+6h (1.7 ± 0.1 \( \mu m^2/\mu m \)) and 12h fetuses (1.9 ± 0.1 \( \mu m^2/\mu m \)) than in their controls (1.3 ± 0.1 \( \mu m^2/\mu m \)). The area of ASM was also greater in 12h+7d fetuses (1.4 ± 0.1 \( \mu m^2/\mu m \)) compared with 117 DGA controls (1.2 ± 0.1 \( \mu m^2/\mu m \), \( P < 0.05 \)). ASM area was not different between control fetuses at 110 and 117 DGA.
In airways that had proliferating ASM cells, there were on average two, and no more than nine, proliferating cells present. The proportion of airways that contained proliferating ASM cells was not significantly different between the 6h/6h and 12h fetuses (21.7 ± 7.5%) and 117 DGA controls (33.3 ± 6.1%). Similarly, there was no significant difference between the 6h/7d fetuses (6.7 ± 3.6%) and 117 DGA controls (4.1 ± 0.1 μm²/μm). In control animals, the collagen area was significantly smaller (P < 0.05) at 117 DGA than at 110 DGA.

**Alveolar attachments.** Because of the hypercellularity of the parenchymal tissue surrounding the bronchioles in 6h+6h and 12h fetuses (3), the number of attachment points could not be calculated. In 12h+7d fetuses, there were significantly fewer attachments per millimeter of Pbm (30.6 ± 0.8) compared with 117 DGA controls (36.3 ± 0.7, P < 0.001).

**Qualitative Analysis of Injured Airways**

Injured airways from 6h+6h and 12h fetuses exhibited differing degrees and types of epithelial damage, and these were the only groups that showed severe epithelial damage (Table 1 and Fig. 1A). The 12h+7d fetuses showed little...
evidence of epithelial damage, with the proportion of injured airways being similar than that in 110 and 117 DGA controls. The majority of injured airways in the 6h/H11001/6h (93%) and 12h groups (97%) showed partial obstruction of the lumen by cellular or acellular debris (Fig. 1A).

### Table 1. Severity of airway injury

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<tr>
<th>Severity Score</th>
<th>Total No. of Airways</th>
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<td>Mild</td>
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<td>Moderate</td>
<td>55</td>
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<td>Severe</td>
<td>46</td>
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<tr>
<td>117 DGA Control</td>
<td>3</td>
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<td>12h + 7d</td>
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Airway injury was qualitatively scored as being mild, moderate, or severe according to the severity of epithelial injury. The airway had mild injury if <45° of the epithelium was detached or absent from the basement membrane, moderate injury if between 45 and 180° of the epithelium was detached or absent, and severe injury if >180° of the epithelium was detached or absent.

### DISCUSSION

Using the novel technique of in utero ventilation, we have investigated the effects of positive-pressure ventilation alone on the bronchiolar airways at a stage of lung development comparable to that of very preterm infants. Previously we showed that brief periods of positive-pressure ventilation of the very immature ovine lung induced persistent alterations in alveolar structure and in the lung interstitium (3). In the present study, we have demonstrated for the first time that even a short period of ventilation (12 h) of the very immature lung is capable of inducing persistent remodeling of the bronchiolar wall and reducing the number of alveolar attachments. Specifically, we have shown that 12 h of mechanical ventilation per se resulted 7 days later in increases, relative to Pbm, in the amount of ASM and collagen; the epithelium was 20% thicker, with greatly increased numbers of proliferating and apoptotic cells. Together, with a reduced number of alveolar attachment points, these changes could contribute to increased airway resistance and decreased lung function in later life. We also have shown that short periods of ventilation can lead to epithelial detachment or loss but that this gross injury can be repaired within 7 days in the absence of further ventilation.

![Fig. 3. Effects of ventilation on epithelial thickness in intact airways. A and B: lung tissue stained with Masson’s Trichrome was used in the calculation of the area of epithelium in intact airways. Bars, 20 μm. Image in A shows the airway epithelium in a representative example of a control fetus at 117 DGA; image in B shows the airway epithelium in a representative example of a 12h + 7d fetus. C: area of the airway epithelium in control fetuses at 110 and 117 DGA and in fetuses ventilated in utero at 110 DGA for 6 h followed by 6 h of recovery, for 12 h with no recovery period, and for 12 h followed by 7 days of recovery. Values are means ± SE; n = 5 for each group. a,b,c P < 0.05, values that do not share a common letter are significantly different from each other.](image-url)
Our study was designed to address the contribution of positive-pressure ventilation to alterations in lung structure that are characteristic of ventilation-induced lung injury, rather than replicating ventilation procedures currently used in the clinic. Our use of high inspiratory pressures and dry gas without the instillation of surfactant was intended to create a ventilation paradigm that would cause lung injury and may be more representative of ventilatory procedures used in the past. The high inspiratory pressures used were necessary to achieve adequate tidal volumes in the very immature fetus, which has incompliant, surfactant-deficient lungs. Despite the use of high pressures, we believe that no localized volutrauma occurred; we made observations in three anesthetized fetuses at the same gestational age (110 DGA), with the fetal chest open to observe

Fig. 4. Epithelial proliferating, apoptotic, and mucin-containing cells in intact airways. The number of proliferating cells (A), apoptotic cells (B), and mucin-containing cells (C) in the airway epithelium are expressed as a percentage of the total number of airway epithelial cells counted in the different treatment groups. The groups comprised control fetuses at 110 and 117 DGA and fetuses ventilated in utero at 110 DGA for 6 h followed by 6 h of recovery, for 12 h with no recovery period, and for 12 h followed by 7 days of recovery. Values are means ± SE; n = 5 for each group. *P < 0.05, values that do not share a common letter are significantly different from each other.

Fig. 5. Smooth muscle and collagen in the airway wall. The area of smooth muscle (A) and collagen fibers (B) was determined in the airway wall in control fetuses at 110 and 117 DGA and in fetuses ventilated in utero at 110 DGA for 6 h followed by 6 h of recovery, for 12 h with no recovery period, and for 12 h followed by 7 days of recovery. The analyses were performed on sections stained with α-smooth muscle actin antibody for the determination of smooth muscle area or with Gordon and Sweet’s reticular fiber stain for the determination of collagen area. Values are means ± SE; n = 5 for each group. *P < 0.05, values that do not share a common letter are significantly different from each other.

In Utero Ventilation Animal Model

Our study was designed to address the contribution of positive-pressure ventilation to alterations in lung structure that are characteristic of ventilation-induced lung injury, rather than replicating ventilation procedures currently used in the clinic. Our use of high inspiratory pressures and dry gas without the instillation of surfactant was intended to create a ventilation paradigm that would cause lung injury and may be more representative of ventilatory procedures used in the past. The high inspiratory pressures used were necessary to achieve adequate tidal volumes in the very immature fetus, which has incompliant, surfactant-deficient lungs. Despite the use of high pressures, we believe that no localized volutrauma occurred; we made observations in three anesthetized fetuses at the same gestational age (110 DGA), with the fetal chest open to observe
the distribution of ventilation, and in each fetus lung expansion and gas distribution appeared to be uniform. We recognize that thoracic restriction in utero may conceivably oppose uniform ventilation, and future studies using radiographic imaging, as we have employed in immature rabbit kits (37, 41), may help to clarify this issue.

Airway Size

The airways that we examined were bronchioles <350 μm in diameter. These small airways are believed to play an important role in determining lung function and in the etiology of lung disease (10, 21, 24). In a study such as ours, it is important to analyze airways of similar size in each treatment group, because differences could affect the interpretation of the results. Because there were no differences between treatment groups in the mean Pbm of airways that were analyzed, and because the frequency distribution of Pbm values was similar between groups, it is likely that we studied similar generations of airways; this implies that the observed alterations in the structural components of the bronchiolar walls were not a result of differences in airway size or generation between groups but were due to the treatments. Because it is not possible to microdissect airways of this size, we cannot establish their generation number. Although it is not known exactly which generations these airways represent, the greater frequency of airways with a Pbm of 300–700 μm suggests that most are small bronchioles including terminal bronchioles.

Bronchiolar Epithelium

Although the epithelial thickness was similar to that of age-matched controls immediately following positive-pressure ventilation, at 7 days after 12 h of ventilation, the epithelium was thicker than in age-matched controls. In control fetuses, epithelial thickness decreased with age; therefore, our findings suggest that the epithelium fails to thin following 12 h of ventilation. Previous studies also have shown increased epithelial thickness following ventilation in preterm baboons (9) and infants with BPD (43); however, in an earlier study of preterm infants, ventilation was not found to alter epithelial height in small airways (18). If it persists into later life, a thicker epithelium could contribute to decreased lung function or an increased risk of airway diseases such as asthma in children who were born very preterm (16). Given that there is now evidence to suggest that the airway epithelium in asthma is fundamentally abnormal, with increased susceptibility to environmental injury and impaired repair (22), it will be interesting to determine whether the thicker epithelium we saw 7 days after 12 h of ventilation persists and predisposes these airways to effects of asthma allergens.

In control fetuses, there was a markedly lower rate of epithelial cell proliferation at 117 DGA than at 110 DGA, which could be causally linked to the age-related thinning of the epithelium. Immediately after 12 h of ventilation, the proportion of epithelial cells undergoing proliferation was ~50% lower than in age-matched controls; however, 7 days later, the rate of proliferation was 18-fold greater than in controls, suggesting that even in the absence of ventilation, abnormal cell proliferation occurs as a result of the earlier ventilatory insult. The increased epithelial cell proliferation may be part of a repair mechanism whereby the epithelium is responding to the reduced proliferation that occurs immediately following positive-pressure ventilation or is replacing the cells that have either been injured or undergone apoptosis immediately after ventilation. Regulation of the cell cycle is acquired through a balance between cell growth and cell death, and cell-cycle checkpoints enable the balance to be maintained. p53, a cell-cycle checkpoint protein that is activated in response to DNA-damaging agents, can induce cell-cycle arrest and/or apoptosis (13, 35). Increased expression of p53 in bronchiolar and alveolar epithelial cells, together with increased apoptosis, has been found in the lungs of ventilated preterm baboons exposed to 100% oxygen (13). Although in the present study the lungs were ventilated with air rather than 100% oxygen, increased apoptosis was observed in bronchiolar epithelial cells immediately after positive-pressure ventilation, indicating that ventilation per se, potentially via shear stress, could induce cell death. Furthermore, a 3.5-fold increase in epithelial cell apoptosis occurred in the absence of continued ventilation, which could possibly impede the normal repair process.

Evidence of increased airway secretory activity has been found in preterm, ventilated infants (18). Similarly, we found that immediately after 12 h of ventilation, the percentage of PAS-positive epithelial cells was increased, suggesting an increase in epithelial mucus content. However, 7 days after ventilation, PAS-positive staining was similar to that of controls, indicating a return to normal secretory function. Our findings suggest that positive-pressure ventilation, at least in the short term, can stimulate mucus production in the small airways of the very immature lung. This may be related to elevated levels of proinflammatory cytokines (6, 12, 29) and could contribute to mucus plugging and airway obstruction.

Airway Smooth Muscle

Positive-pressure ventilation increased α-SMA-positive staining in the bronchiolar wall compared with controls, indicating a greater amount of ASM. This difference was evident 7 days later, indicating that as little as 12 h of ventilation can persistently affect the ASM. Our findings are consistent with previous studies that found increased muscularization of distal airways in ventilated immature lungs in infants (30, 39) and preterm sheep (1); indeed, increased bronchiolar ASM is a hallmark of BPD (8). Because ASM is critical in the control of airway resistance and reactivity, a persistent increase in the amount of ASM could impair later lung function and increase bronchial reactivity. It is thought that the shear stress and repeated cyclic tissue stretch exerted by mechanical ventilation could stimulate airway muscularization; however, previous studies have been unable to determine whether the increased ASM is due to cellular hyperplasia or hypertrophy (1). We have demonstrated for the first time that ASM cell proliferation and apoptosis are not altered following positive-pressure ventilation, indicating that ventilation likely increases ASM via smooth muscle cell hypertrophy rather than by hyperplasia.

Because it was difficult to measure the number of ASM cells within each airway section, we expressed the number of proliferating and apoptotic ASM cells as a percentage of the number of airways analyzed. This, however, does not detract from our conclusion that ASM underwent cellular hypertrophy; because there were no apparent differences in ASM cell pro-
liferation or apoptosis between control and ventilated fetuses, cellular hypertrophy appears to be the only explanation for the increase in ASM area in the ventilated fetuses.

**Bronchiolar Collagen**

Collagen types I and III are predominantly associated with mesenchymal connective tissue in the lung (23, 42). High tensile strength is a key mechanical property of collagen, and its ability to resist distending forces is essential for volume-limiting properties of the lung. Increased and abnormal collagen content and deposition in the alveolar walls have been demonstrated in preterm infants with BPD (7, 20, 23, 42); however, bronchiolar collagen content following positive-pressure mechanical ventilation does not appear to have been studied previously. We found that 12 h of ventilation acutely decreased the amount of bronchiolar collagen. Raised levels of matrix metalloproteinases (MMPs) have been found in the lungs of baboon neonates with BPD (40); since MMPs can degrade collagens, this could offer an explanation for the reduction in airway collagen immediately after 12 h of ventilation. Importantly, 7 days after 12 h of ventilation, the amount of bronchiolar collagen was greater than in age-matched controls. Changes in the extracellular matrix that is coupled with ASM cells are thought to alter the physical properties of ASM cells (44), and increased collagen content could increase airway wall stiffness, thereby contributing to impaired lung function (5).

**Alveolar Attachments**

Alveolar-bronchiolar attachments play an important role in structural support of the small airways (36). To date, no study has investigated alveolar attachments in preterm neonates who developed BPD or in animal models of BPD. We found a decrease in the number of attachments 7 days following 12 h of ventilation; this is likely explained by the disruption in alveolarization after positive-pressure mechanical ventilation (3). An important potential consequence of fewer alveolar attachments is reduced airway tethering, which is likely to contribute to airway narrowing and reduced lung function (36).

**Epithelial Injury**

Consistent with previous studies (11, 28, 39), we found that positive-pressure ventilation can cause epithelial detachment and denudation and also can cause debris to accumulate within the airway lumen. Ventilation for 6 and 12 h injured the epithelium of more than 10 and 30% of the bronchioles, respectively; however, 7 days later, very few airways remained injured. Our findings show that the duration of positive-pressure ventilation plays a role in the extent of damage to the airways and that repair mechanisms can ameliorate ventilator-induced injury in the absence of continued ventilation. Mechanisms leading to the detachment and loss of epithelium are unknown but are likely to include shear stress and sloughing of epithelial cells following their death; the latter is likely, because there is an increase in epithelial cell apoptosis following positive-pressure ventilation. Since the epithelium is the interface between the external and internal environment, it is likely to be one of the first components of the airway wall to become injured by positive-pressure ventilation. In our study, the use of unhumidified gas could have contributed to the epithelial injury as well as the increase in epithelial thickness observed following positive-pressure ventilation. Inflammation also may play a role in denudation of the epithelium, because many of the airways that had either complete or partial epithelial denudation also presented with inflammatory cells within the lumen.

Although luminal debris was commonly seen after ventilation, the type of debris appeared to be dependent on the duration of positive-pressure ventilation. Animals ventilated for 6 h often presented with inflammatory cells within the lumen; however, those ventilated for 12 h typically presented with partial obstruction of the lumen with an acellular substance. The acellular substance is thought to be hyaline membrane, because these airways appear similar to those from preterm human lungs affected by hyaline membrane disease (34). Hyaline membrane is generally thought to be caused by prolonged periods of mechanical ventilation; however, our study has shown that this can occur within as little as 12 h. As well as luminal debris blocking or narrowing the airways, its presence could generate further airway damage by promoting inflammation.

**Conclusions**

Short periods of positive-pressure ventilation during the canalicular stage of lung development, in the absence of other factors that accompany very preterm birth, can injure and alter the structure of the small conducting airways with some changes persisting for at least 7 days. As little as 12 h of ventilation can induce profound and persistent remodeling of the bronchioles, including changes in the epithelium, collagen, and ASM, which, together with alterations in the lung parenchyma (3) and a reduced number of alveolar attachments, could underlie impaired lung function and an increased risk of obstructive lung disease later in life. Further studies are required to determine whether the effects that we have observed persist throughout life and are sufficient to adversely affect lung function.

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**GRANTS**

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