Nasal Ventilation Alters Mesenchymal Cell Turnover and Improves Alveolarization in Preterm Lambs

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Rationale: Bronchopulmonary dysplasia (BPD) is a frequent cause of morbidity in preterm infants that is characterized by prolonged need for ventilatory support in an intensive care environment. BPD is characterized histopathologically by persistently thick, cellular distal airspace walls. In normally developing lungs, by comparison, remodeling of the immature parenchymal architecture is characterized by thinning of the future alveolar walls, a process predicated on cell loss through apoptosis.

Objectives: We hypothesized that minimizing lung injury, using high-frequency nasal ventilation to provide positive distending pressure with minimal assisted tidal volume displacement, would increase apoptosis and decrease proliferation among mesenchymal cells in the distal airspace walls compared with a conventional mode of support (intermittent mandatory ventilation).

Methods: Accordingly, we compared two groups of preterm lambs: one group managed by high-frequency nasal ventilation and a second group managed by intermittent mandatory ventilation. Each group was maintained for 3 days.

Measurements and Main Results: Oxygenation and ventilation targets were sustained with lower airway pressures and less supplemental oxygen in the high-frequency nasal ventilation group, in which alveolarization progressed. Thinning of the distal airspace walls was accompanied by more apoptosis, and less proliferation, among mesenchymal cells of the high-frequency nasal ventilation group, based on morphometric, protein abundance, and mRNA expression indices of apoptosis and proliferation.

Conclusions: Our study shows that high-frequency nasal ventilation preserves the balance between mesenchymal cell apoptosis and proliferation in the distal airspace walls, such that alveolarization progresses.

Keywords: alveolar formation; bronchopulmonary dysplasia; chronic lung disease of prematurity; morphometry; stereology

Respiratory distress syndrome is an important cause of morbidity and mortality among preterm neonates (1–3). The principal long-term morbidity is bronchopulmonary dysplasia (BPD) (3). Intubation and conventional mechanical ventilation are thought to increase the likelihood of BPD (4–6), which is characterized histopathologically by alveolar simplification (7, 8). Accumulating anecdotal evidence suggests that the use

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Growing clinical evidence suggests that nasal continuous positive airway pressure (nCPAP) reduces the risk for premature infants to develop bronchopulmonary dysplasia, but this remains controversial. The biological basis for differences between the effects of nCPAP and standard mechanical ventilation are uncertain.

What This Study Adds to the Field

We report that high frequency nasal ventilation, which is similar to nCPAP, alters the pattern of apoptosis and proliferation of mesenchymal cells in the distal airspace walls in the immature lung, which may account for differences in outcomes.

of continuous positive airway pressure (CPAP), as compared with the use of conventional mechanical ventilation, reduces the incidence of BPD (4). Experimental evidence from different animal models has shown that, compared with conventional mechanical ventilation, CPAP improves lung volumes (9, 10) and permits normal alveolar progression (11). However, the mechanisms for the apparent better outcomes are not known.

During the later stages of normal lung development (canalicular, saccular, and alveolar), the mesenchymal compartment becomes thinner in the parenchymal regions of the lung, where the distal airspaces form. Based on morphologic analysis of normal postnatal lung development in rats, mesenchymal thinning results in part from reduced fibroblast cell number, through the process of apoptosis (12). In contrast to normal lung development, lung architecture in BPD is characterized in part by lung mesenchyme that remains thick and cellular (8, 13–16), suggesting that expected mesenchymal cell loss is disrupted after preterm birth accompanied by prolonged conventional mechanical ventilation. Although apoptotic mesenchymal cells have been identified in the lungs of preterm neonates with BPD (16–18), the impact of ventilation mode on apoptosis of mesenchymal cells, and associated thinning of the distal airspace wall, has not been determined. Because (1) the balance between apoptosis and proliferation is important for formation of thin-walled alveoli, and (2) the histopathology of BPD in preterm neonates who have been managed by conventional mechanical ventilation is characterized in part by thick and cellular mesenchyme in the distal airspace walls, we hypothesized that apoptosis and proliferation of mesenchymal cells would be different, depending on ventilation mode. We compared those outcomes between preterm lambs that were managed for 3 days by intermittent mandatory ventilation (IMV) or high-frequency nasal ventilation (HFNV). HFNV maintains functional residual lung volume by positive distending pressure, with minimal assisted tidal volume displacement. We used HFNV

(Received in original form February 29, 2008; accepted in final form June 10, 2008)

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Supported by NIH grants HL62875, HL56401, HL07744, HD01410, and the Children's Health Research Center in the Department of Pediatrics.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Crit Care Med Vol 178, pp 407–418, 2008

Originally Published in Press as DOI: 10.1164/rccm.200802-359OC on June 12, 2008

Internet address: www.atsjournals.org

because it provided effective ventilation beyond 3 to 4 hours, whereas bubble nasal CPAP did not, secondary to nose length. Accordingly, we used our preterm lamb model to identify the effect of ventilation mode on the regulation of mesenchymal thickness and mesenchymal cell apoptosis and proliferation. The results show that HFNV preserves the balance between mesenchymal cell apoptosis and proliferation in the distal airspace walls, such that alveolarization progresses.

METHODS

Surgical Preparation and Management of Preterm Lambs

The protocols adhered to APS/NIH guidelines for humane use of animals for research, and were prospectively approved by the IACUC at the University of Utah Health Sciences Center. The methods for the chronic ventilation model using preterm lambs have been reported (8, 19–24). Details are presented in the online supplement. Briefly, time-pregnant ewes that carried single or twin fetuses at 130 to 132 days of gestation (term $\sim$ 150 d of gestation) were used. The pregnant ewes were given an intramuscular injection of dexamethasone phosphate (6 mg; Vedco, Inc., St. Joseph, MO), 24 hours before operative delivery. On the day of delivery, the ewes received intramuscular ketamine hydrochloride (10–20 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA), followed by inhalation anesthesia with 1% isoflurane (Abbott Laboratories, North Chicago, IL). We exposed each fetus by midline hysterotomy, placed catheters in the common carotid artery and external jugular vein, and intubated them with a cuffed endotracheal tube (3.5–4.0 French), through which 10 ml of lung liquid was aspirated and replaced with Survanta (2.5 ml, NDC 0074-1040-08; Ross Products Division, Abbott Laboratories, Columbus, OH). For the fetuses assigned to high-frequency nasal ventilation (described below), an uncuffed oral/nasal true Murphy tube (3.0–4.0 mm inner diameter; 13 cm length) was also inserted through a nostril to reach the mid-length of the nasal cavity (5–6 cm of the $\sim$ 10-cm-long cavity in fetal lambs at $\sim$ 132 d gestation; data not shown). Lidocaine (1% solution; Hospira, Inc, Lake Forest, IL) was injected subcutaneously along the nostril to minimize pain and discomfort. Each fetus was removed from the uterus and its umbilical cord was ligated and cut.

During resuscitation, all of the preterm lambs were managed by IMV, with warmed and humidified 100% oxygen (O_2) (Bird VIP ventilator, model 15215; Bird Products, Palm Springs, CA). Respiratory rate was 60 breaths/minute, inspiratory time was 0.3 seconds, peak inspiratory pressure (PIP) was adjusted to attain a target Pa_{CO_2} between 45 and 60 mm Hg and pH between 7.25 and 7.35, and positive end-expiratory pressure (PEEP) was set at 8 cm H_2O . Target expiratory tidal volume, measured by the ventilator, was 5 to 7 ml/kg/breath. Survanta (2.5 ml) was administered again at 5 to 10 minutes of life. The lambs were shifted from side to side to improve the distribution of Survanta. Subsequently, the concentration of inspired O_2 was decreased to attain a target Pa_{O_2} between 60 and 80 mm Hg. All of the preterm lambs were treated intravenously, within 30 minutes of delivery, with a loading dose of caffeine citrate to stimulate ventilatory drive (25) (15 mg/kg, given over 2 h; Mead, Johnson and Co., Evansville, IN), followed by maintenance treatment with 5 mg/kg every 24 hours.

We used two ventilation modes: (1) IMV or (2) HFNV. For the preterm lambs assigned to HFNV, weaning from IMV was begun at 2 hours of life. Weaning was successful when the preterm lambs consistently breathed spontaneously while the ventilator circuit was disconnected from the endotracheal tube. At that time, the endotracheal tube was removed and the ventilator circuit from a high-frequency flow-interrupter device (Percussionaire Inc., Sand Point, ID) was attached to the tube in the nose, with initial ventilator settings of amplitude (20–25 cm H_2O), mean pressure (8–12 cm H_2O), end-expiratory pressure (5–7 cm H_2O), and rate (10 Hz). The ventilator circuit included a gas humidifier. Adjustments were made to the ventilator settings to reach the target ranges for Pa_{O_2} and Pa_{CO_2} . We matched target Pa_{O_2} (60–80 mm Hg) to that for the IMV group by adjusting Fi_{O_2} . We matched target Pa_{CO_2} (45–60 mm Hg) to that for the IMV group by adjusting amplitude on the Percussionaire. Intratracheal pressure was measured using a catheter passed through the mouth and vocal cords to the trachea (Unisensor USA, Inc., Hampton, NH).

HFNV was used because clinical and experimental animal studies suggest that early nasal CPAP is associated with lower incidence of BPD (4), less inflammation (10), improved lung volumes (9, 10), and greater alveolar complexity (11, 26) compared with IMV (7, 8). Initially, we tried bubble nasal CPAP (10, 27). However, beyond 3 to 4 hours of continuous bubble nasal CPAP, Pa_{CO_2} rose and pH fell to unphysiologic levels, despite treating the preterm lambs with antenatal corticosteroids to accelerate lung development, exogenous surfactant to increase lung compliance, and caffeine citrate to increase respiratory drive. The only prior use of HFNV we are aware of is a small observational study in preterm neonates failing nasal CPAP reported by van der Hoeven and colleagues (28). They applied HFNV with the Infant Star 950 ventilator via a single nasopharyngeal tube in a study group that was quite diverse with respect to gestational and postnatal age. Interestingly, there was significant improvement in ventilation, but mean nasal airway pressure was slightly higher in the HFNV-treated neonates.

We chose an experimental duration of 3 days because other studies from our laboratory demonstrated that tropoelastin mRNA expression was up-regulated at 3 days (24). Two preterm lambs were studied simultaneously. To avoid selection bias, the order of ventilation mode was alternated so that consecutive preterm lambs were not managed by the same ventilation mode.

Preterm lambs were kept prone in a veterinary sling mounted on a heated bed. Saline and dextrose solutions were administered intravenously, as were antibiotics and sedatives (pentobarbital sodium at frequent intervals; Abbott Laboratories, North Chicago, IL, and buprenorphine hydrochloride, 5 μ g/kg every 3 h; Reckitt and Colman Pharmaceuticals, Richmond, VA). The preterm lambs assigned to IMV received 3 to 5 mg/kg of pentobarbital as needed to minimize discomfort associated with endotracheal intubation. Nevertheless, the lambs maintained ability to respond to external stimulation as evidenced by their response to tracheal suction. The preterm lambs assigned to HFNV, on the other hand, received 1 to 2 mg/kg of pentobarbital so that they breathed spontaneously. Vascular pressures and heart rate were continuously recorded (V6400; SurgVet, Inc., Waukesha, WI). Arterial blood gases and pH, and plasma concentrations of glucose and electrolytes, were measured hourly. Plasma concentrations of total protein and hematocrit were measured at 6-hour intervals. An orogastric feeding tube was used for enteral feedings, using ewe's fresh colostrum, beginning at 8 hours of life (3–5 ml/kg every 2 h). The feeding tube was withdrawn between feedings. The volume of colostrum was increased gradually by 5-ml increments, as tolerated, to attain a goal of 60 Kcal/kg/day of total energy substrate. We monitored total fluid intake (saline, dextrose, and milk) and output (urine and stool), and made adjustments to maintain fluid homeostasis, as indicated by urine output ($>$ 1–2 ml/kg/h) and blood pressure.

Chest radiographs were taken daily to assess lung inflation volume and to identify atelectasis. None of the preterm lambs developed air leaks. Indices of infection were monitored by daily leukocyte total and differential cell counts, and core body temperature. If the lambs became hypotensive (systemic arterial blood pressure $<$ 40 mm Hg), a series of steps was taken in the following order: intravenous saline bolus and fresh frozen plasma. None of the preterm lambs required treatment with pressors.

At the end of each 3-day study, final blood samples were collected before the preterm lambs were treated with heparin (1,000 U, intravenously). The HFNV group was reintubated and managed for less than 1 minute, with the same ventilator and settings during HFNV. All lambs were killed, using 60 mg/kg pentobarbital sodium solution, intravenously (Ovation Pharmaceuticals, Inc., Deerfield, IL). The chest was opened, the trachea was ligated at end-inspiration (to minimize atelectasis), and the lungs and heart were removed as a block. The whole left lung was insufflated with 10% buffered neutral formalin (static pressure of 25 cm H_2O). Fixed-lung displacement volume was measured by suspension in formalin before the lung was stored in fixative at 4°C for 24 hours. We used the cardiac lobe for transmission electron microscopy. The right lung was cut into approximately 1 cm³ pieces that were snap-frozen in liquid nitrogen and stored at $-80^{\circ}C$ until prepared for immunoblot and quantitative real-time PCR analyses.

TABLE 1. OVINE PRIMER AND PROBE SEQUENCES

	Forward 5' Primer Sequence	Reverse 5' Primer Sequence	Probe 5'
p53	ACTGGAAGACTCTCGTGGTAACCT	CCCAGGACAGGCACAAACAC	CACCTCAAAGTGCTCCGTCACAG
Caspase 3	GCAGTTTTATTGCGTGCTTCTAA	GGTCCATTGGTCCAAAAATGA	CCATGGTGAAGAAGG
TGF- β	TCCCTACATCTGGAGCCTGG	CCGGGTTGTGCTGGTTGTAC	CACACAGTACAGCAAGGCTCTGGCCC
c-Myc	CCAGCAGCGACTCTGAGGA	CCGATTCTGACCTTTTGGCA	TGATGTTGTCTCTGTGAAAAAGGCCAAC
GAPDH	GAGCTCAACGGGAAGCTCAC	TGATCTCATCATACTTGGCAGGTT	TGGATCTGACCTGCCGCTCGGA

To appropriately compare the impact of ventilation strategy on lung development, we also prepared lung tissue from three control groups: two groups of fetal lambs and one group of term newborn lambs. One fetal group was delivered at a fetal age of approximately 132 days gestation (FA132), and thus matched the gestation age when the preterm lambs were delivered. The other fetal group was delivered at a fetal age of approximately 136 days gestation (FA136), and therefore matched the gestation age when the preterm lamb studies ended. The fetal lambs were not allowed to breathe before they were killed. The term newborn lambs were allowed to spontaneously breathe after birth at term (~ 150 d) before killing, within 24 hours of birth. The latter control group was included because alveolarization is approximately 80% complete at term gestation in sheep (29).

Morphometry for Alveolar Secondary Septation and Distal Airspace Wall Thickness

We assessed alveolar secondary septation and distal airspace wall thickness by morphometry, using methods previously reported by our group (8, 20, 21, 23, 30).

Histochemistry and Immunohistochemistry, and Morphometry of Labeled Cells

We used histochemistry, immunohistochemistry, and double immunofluorescence to identify apoptotic and proliferating cells in lung tissue sections (31). We localized TUNEL, p53, Bax, cleaved caspase-3, and PCNA protein, as well as specific markers of cells of mesenchymal origin (desmin and vimentin).

Immunoblot

Homogenates of frozen samples of peripheral, whole-lung tissue (devoid of visceral pleura and central airways/vessels/connective tissue) were used to quantify the relative abundance of cleaved caspase-3 and PCNA proteins (21).

Real-Time PCR

Peripheral, whole-lung tissue homogenates also were used for analysis of p53, caspase-3, TGF- β , and c-Myc mRNA expression (Table 1) (32–34).

Data Analysis

Data are reported as mean $\pm$ 1 SD. Statistical tests were ANOVA followed by nonparametric *post hoc* test (Fisher's PLSD test). We accepted $P < 0.05$ as indicative of statistical differences (35).

RESULTS

HFNV Improved Oxygenation

The characteristics of all of the lambs are summarized in Table 2. No differences were detected between the two groups of ventilated preterm lambs.

Respiratory parameters are summarized in Table 3. Spontaneous respiratory rate in the HFNV group was 40 to 50 breaths/minute. Respiratory rate in the IMV group was set at 60 breaths/minute. Ventilation (P_{aCO_2}) was targeted for 45 to 60 mm Hg for both ventilation groups, and no differences were found between them on Days 1, 2, or 3. Similarly, no differences in pH were observed. In a subset of the HFNV group of preterm lambs, we measured intratracheal pressure. Mean intratracheal pressure averaged 2.3 ± 2.1 cm H₂O (mean $\pm$ SD; range +6.1 to -1.0 cm H₂O) on Days 1 and 2 (measurements were not made on Day 3). The intratracheal high-frequency amplitude pressure change was measured during 20-millisecond intervals. The mean pressure amplitude averaged 0.37 ± 0.23 cm H₂O (range, 0.03 to 0.90 cm H₂O). For the IMV group, mean airway pressure was 13 ± 2 cm H₂O, peak inspiratory pressure was 25 ± 8 cm H₂O, and mean expiratory tidal volume was 5 ± 1 ml/kg across the 3-day study period. Target oxygenation was 60 to 80 mm Hg for both ventilation groups, and no differences were found between them on Days 1, 2, or 3. Maintaining that target required similar FI_{O_2} at the end of 24 hours for both groups. On Days 2 and 3 of ventilation, lower FI_{O_2} was required for the HFNV group compared with the IMV group ($P < 0.05$). Tidal volume and functional residual volume were not measured in the HFNV group; however, effective oxygenation at lower FI_{O_2} suggested that functional residual volume was better for the HFNV group.

HFNV Permitted Alveolarization

Alveolar secondary septation was different between the HFNV and IMV groups of preterm lambs (Figure 1). Terminal respiratory units (TRUs) and distal airspaces were smaller and more uniformly shaped, had more and longer alveolar secondary septa, and had thinner distal airspace walls in the preterm lambs that were managed by HFNV compared with IMV. Those qualitative features were borne out by morphometric

TABLE 2. CHARACTERISTICS OF PRETERM LAMBS THAT WERE VENTILATED FOR 3 DAYS AND GESTATION CONTROL LAMBS (MEAN $\pm$ SD)

Characteristic	Intermittent Mandatory Ventilation (n = 8)	High-Frequency Nasal Ventilation (n = 8)	FA132 (n = 4)	FA136 (n = 4)	Term Newborn (n = 4)
Male/Female	5/3	4/4	2/2	3/1	2/2
Gestation age at delivery, d	132 $\pm$ 1*	132 $\pm$ 1*	132 $\pm$ 1*	136 $\pm$ 1	146 $\pm$ 1†
Weight at birth, g	3,969 $\pm$ 242†	3,746 $\pm$ 459	3,420 $\pm$ 324	4,278 $\pm$ 193*	5,729 $\pm$ 390 #
Weight at death, g	3,779 $\pm$ 346*	3,614 $\pm$ 499	3,420 $\pm$ 324	4,278 $\pm$ 193	5,729 $\pm$ 390

Definition of abbreviations: FA132, lambs delivered at a fetal age of approximately 132 d; FA136, lambs delivered at a fetal age of approximately 136 d.

* $P < 0.05$ compared with FA136 group by ANOVA and Fisher's PLSD *post hoc* test.

† $P < 0.05$ compared with all other groups by ANOVA and Fisher's PLSD *post hoc* test.

$P < 0.05$ compared with FA132 group by ANOVA and Fisher's PLSD *post hoc* test.

TABLE 3. RESPIRATORY PARAMETERS FOR PRETERM LAMBS THAT WERE VENTILATED FOR 3 DAYS (MEAN $\pm$ SD)

Parameter	Intermittent Mandatory Ventilation (n = 8)	High-Frequency Nasal Ventilation (n = 8)
PIP, cm H ₂ O		
24 h	26 $\pm$ 3	—
48 h	24 $\pm$ 7	—
72 h	26 $\pm$ 10	—
PEEP, cm H ₂ O		
24 h	8 $\pm$ 1	—
48 h	7 $\pm$ 1	—
72 h	7 $\pm$ 1	—
MAP, cm H ₂ O		
24 h	13 $\pm$ 2	—
48 h	13 $\pm$ 2	—
72 h	14 $\pm$ 3	—
Intratracheal pressure, cm H ₂ O*		
24 h	—	2.9 $\pm$ 2.4
48 h	—	1.6 $\pm$ 1.8
72 h	—	—
Pa _{CO₂} , mm Hg		
24 h	55 $\pm$ 14	58 $\pm$ 17
48 h	49 $\pm$ 6	57 $\pm$ 6
72 h	60 $\pm$ 11	58 $\pm$ 7
pH		
24 h	7.30 $\pm$ 0.09	7.29 $\pm$ 0.10
48 h	7.38 $\pm$ 0.08	7.34 $\pm$ 0.04
72 h	7.34 $\pm$ 0.11	7.37 $\pm$ 0.06
F _{IO₂} , %		
24 h	71 $\pm$ 11	59 $\pm$ 28
48 h	59 $\pm$ 17	41 $\pm$ 8†
72 h	48 $\pm$ 12	34 $\pm$ 6†
Pa _{O₂} , mm Hg		
24 h	64 $\pm$ 7	76 $\pm$ 17
48 h	71 $\pm$ 17	76 $\pm$ 20
72 h	82 $\pm$ 14	83 $\pm$ 16

Definition of abbreviations: MAP = mean airway pressure; PEEP = positive end-expiratory pressure; PIP = peak inspiratory pressure.

* Measured in 4 of the 8 preterm lambs.

† $P < 0.05$ compared with IMV by Mann-Whitney U-test.

data for each set of preterm lambs (Figure 2). The HFNV group had greater radial alveolar count ($P < 0.05$) and volume density for alveolar secondary septa ($P < 0.05$), and narrower distal airspace walls ($P < 0.05$), compared with the IMV group. Another difference between the two groups was the lungs of the HFNV group were more homogeneously inflated compared with the IMV group. In the latter group, areas of airspace distension were mixed with areas of microatelectasis.

We also compared the histologic appearance and morphometric measurements for the preterm lambs to those for the gestation control lambs (their characteristics are summarized in Table 2). We made the latter comparison to determine whether management of preterm lambs by HFNV resulted in a rate of alveolarization that reflected normal gestational development *in utero* or was accelerated, given that alveolarization was structurally more advanced in the HFNV group than in the IMV group. Histologically, TRUs and distal airspaces were small and uniformly shaped in the fetal gestation control groups

Figure 1. Histology of lung tissue from preterm lambs ventilated for 3 days by intermittent mandatory ventilation (IMV; *a* and *c*) or high-frequency nasal ventilation (HFNV; *b* and *d*). Each row of panels is the same magnification (refer to corresponding scale bars). Gestation control fetal lambs were (1) delivered at the gestation age that the preterm lambs were delivered (FA132) or (2) delivered at the gestation age when the preterm lamb studies were completed (FA136). The terminal respiratory unit (TRU) in a preterm lamb managed by HFNV (*b* and *d*) has smaller and more uniform distal airspaces, more and thinner alveolar secondary septa (arrowhead in *a* and *b*), and thinner distal airspace walls (arrow in *c* and *d*) than the TRU in a preterm lamb managed by IMV (*a* and *c*). The TRU in the preterm lamb managed by HFNV is structurally similar to the TRU in the FA136 gestation control lamb (*f* and *h*). On the other hand, the TRU in the preterm lamb managed by IMV (*a* and *c*) is structurally similar to the TRU in the FA132 gestation control lamb (*e* and *g*).

(Figure 1), neither of which was allowed to breathe before their lungs were insufflated with fixative. By comparison, the TRUs and distal airspaces in the IMV group of preterm lambs were distended widely (Figure 1). Greater magnification of the distal airspace walls revealed that they were thicker and more cellular in the FA132 gestation control group than the FA136 gestation control group (Figures 1g and 1h, respectively). The breadth and cellularity of the distal airspace walls in the IMV group of preterm lambs

Figure 2. Summary of morphometric measurements (mean $\pm$ SD) made in lung tissue sections from the groups of preterm lambs managed by IMV (open bars) or HFNV (solid bars) ($n = 8/\text{group}$). Radial alveolar count (a) and volume density of secondary septa (b) were significantly greater, whereas distal airspace wall thickness (c) was significantly narrower, in the HFNV group than in the IMV group. Results are compared with results for (1) fetuses delivered at the

gestation age when the preterm lambs were delivered (fetal start, FS; $n = 4$), (2) fetuses delivered at the gestation age when the preterm lamb studies were completed (fetal end, FE; $n = 4$), and (3) term newborn (TN) lambs ($n = 4$). Indices of alveolar secondary septation (radial alveolar count [a], volume density of secondary septa [b], and distal airspace wall thickness [c]) for the HFNV group of preterm lambs were comparable to the corresponding structural features in either the FE or TN gestation control groups. By comparison, the morphometric attributes of the IMV group of preterm lambs were comparable to those of the FS gestation control group. Statistical comparisons were by ANOVA and Fisher's PLSD *post hoc* test. Key for statistical differences ($P < 0.05$): *different from IMV; #different from FS; @different from TN.

(compared with Figures 1c and 1g, respectively), whereas the appearance of the distal airspace walls in the FA136 group appeared like that in the HFNV group of preterm lambs (Figures 1d and 1h, respectively). Likewise, morphometry showed that alveolarization in the IMV group of preterm lambs remained the same as the FA132 gestation control group, whereas alveolarization in the HFNV group of preterm lambs was between the FA136 and term-newborn gestation control groups (Figure 2).

HFNV Revealed a Mechanism for Thinner Distal Airspace Walls

To test the hypothesis that apoptosis was greater, and proliferation less, among the mesenchymal cells in the distal airspace walls of the HFNV group compared with the IMV group, we stained lung tissue sections for histochemical or immunohistochemical markers of apoptosis (TUNEL, p53, Bax, and caspase-3) and proliferation (PCNA) (Figure 3). Nuclei of mesenchymal cells in the distal airspace walls were prominently stained for TUNEL, p53, Bax, and cleaved caspase-3 in lung tissue sections from the HFNV group compared with the IMV group. Conversely, nuclei of mesenchymal cells in the distal airspace walls were minimally stained for PCNA in lung tissue sections from the HFNV group compared with the IMV group. The identity of mesenchymal cells was confirmed by double immunofluorescence staining, by combining an antibody marker of apoptosis (cleaved caspase 3) or cell proliferation (PCNA) with an antibody directed against markers of cells of mesenchymal origin (desmin: smooth muscle intermediate filament cell marker; or vimentin: mesenchymal cell intermediate filament marker). Cleaved caspase 3 or PCNA/desmin double immunolabeling (Figure 4) as well as cleaved caspase 3 or PCNA/vimentin double immunolabeling (Figure 5) showed that cells of mesenchymal origin (stained green) in the interstitial space of the distal airspace walls had red nuclei, indicating that the cells were apoptotic or proliferating, respectively. The same immunolabeling patterns were detected in the fetal and term-newborn gestation control groups (Figures 4 and 5). Airspace epithelial cells (endodermal origin) were not double-labeled. Capillary endothelial cells may have been labeled in the airspace walls by the anti-vimentin antibody but the identity of capillary endothelial cells was not specifically determined.

We used morphometric methods to quantify cell labeling, based on the single-immunostained tissue sections. For this morphometric analysis, we used the number of airspace epithelial cells as the reference space for the mesenchymal cell results. That was important because architecture and distension of the

TRUs was different between the two groups of ventilated preterm lambs (refer to Figure 1). Therefore, using the number of airspace epithelial cells for the reference space avoided impacting the results by differences in parenchymal architecture. The morphometric results (Figure 6) revealed that the number of TUNEL-labeled mesenchymal nuclei (cells) per 100 airspace epithelial cells, as an index of mesenchymal cell apoptosis, was significantly greater in the preterm lambs that were managed by HFNV compared with IMV ($P < 0.05$). Conversely, the number of PCNA-labeled mesenchymal nuclei (cells) per 100 airspace epithelial cells, as an index of mesenchymal cell proliferation, was significantly less in the preterm lambs that were managed by HFNV compared with IMV ($P < 0.05$). The analysis also showed that, once again, the HFNV group of preterm lambs had apoptotic and proliferation indices comparable to the FA136 gestation control group. By comparison, the IMV group of preterm lambs had an apoptotic index that was the same as the FA132 gestation control group, whereas that group's proliferation index was greater than all of the gestation control groups.

Based on the immunohistochemical and double immunofluorescence analyses, which were used to identify the cell types that were apoptotic or proliferating in the lung, we also quantified biochemical and molecular markers of apoptosis and proliferation, using homogenates of peripheral whole-lung tissue from the same lambs' lungs. Immunoblot analysis showed significantly greater abundance of the pro-apoptotic protein, cleaved caspase-3, in the HFNV group compared with the IMV group ($P < 0.05$; Figure 7a). Conversely, PCNA protein abundance was significantly less in the HFNV group compared with the IMV group ($P < 0.05$; Figure 7b). Real-time RT-PCR showed that expression of mRNA for p53 and caspase-3, gene product markers for apoptosis, was significantly greater in the HFNV group compared with the IMV group ($P < 0.05$; Figures 7c and 7d). Conversely, once again, expression of mRNA for TGF- β and c-Myc, gene product markers that are consistent with cell proliferation, was significantly less in the HFNV group compared with the IMV group ($P < 0.05$; Figures 7e and 7f).

DISCUSSION

Preterm birth followed by prolonged ventilation with supplemental oxygen frequently results in BPD, which is characterized histopathologically as alveolar simplification. A feature of the simplification is thick, cellular mesenchyme in the wall of the distal airspaces. However, the mechanisms that lead to that mesenchymal pathology are not known. The present study demonstrates that HFNV allows the normal balance between

Figure 3. Histochemistry and immunohistochemistry of lung tissue from preterm lambs ventilated for 3 days by IMV (a, c, e, g, i) or HFNV (b, d, f, h, j). All of the panels are the same magnification; scale bar is 500 μ m. More TUNEL-stained nuclei of mesenchymal cells (*thin arrows in b*) are evident in the lung tissue section from a preterm lamb managed by HFNV compared with another preterm lamb managed by IMV (a). The *bold arrow* in each panel identifies the region illustrated at greater magnification in the corresponding inset image. DAS, distal airspace. Lung tissue sections from the preterm lamb managed by HFNV also have more immunostain for p53 (d), Bax (f), and cleaved caspase 3 (h) among nuclei of mesenchymal cells compared with the other preterm lamb that was managed by IMV (c, e, and g, respectively). Conversely, the lung tissue section from the preterm lamb managed by HFNV has less immunostain for PCNA (j) among nuclei of mesenchymal cells compared with the other preterm lamb that was managed by IMV (i).

apoptosis and proliferation of mesenchymal cells, whereas IMV disrupts that balance, at 3 days of continuous ventilation. More specifically, IMV significantly decreases apoptosis, and increases proliferation, of mesenchymal cells in the wall of the distal airspaces. The latter findings suggest that specific molecular mechanisms regulating cell number are dependent on the mode of ventilation.

The mechanism responsible for allowing effective ventilation and oxygenation during 3 days of HFNV remains unclear, but may include application of positive airway pressure, increased dead-space washout similar to that observed with tracheal gas insufflation, and reduced work of breathing. Although intranasal airway pressure closely approximated the delivered distending pressure during HFNV of the preterm lambs, there was significant attenuation of pressures to the level of the trachea,

where mean intratracheal pressure (~ 2 cm H_2O) and mean high-frequency pressure amplitude ($\sim \pm 0.4$ cm H_2O) were low. In contrast, mean airway pressure was significantly greater in the IMV group (~ 13 cm H_2O) for the same level of $PaCO_2$. Though we did not measure intratracheal pressures during the application of IMV, a prior study has shown that, with a cuffed endotracheal tube in place, intratracheal measured pressures closely approximate the ventilator-delivered pressures (36). Tracheal gas insufflation has been shown to adequately support ventilation and oxygenation at low airway pressures in neonates with respiratory disease and other pathologic settings (37). An element of increased dead-space gas washout may occur with HFNV because early in the use of HFNV in the preterm lambs, an element of “conventional” nasal positive pressure ventilation was probably present. Previous studies found that nasal positive-

Figure 4. Double immunofluorescence localization, using lung tissue from preterm lambs ventilated for 3 days by IMV or HFNV, as well as fetal controls (FA132 and FA136). All of the panels are the same magnification; scale bar is 25 μm. Red color is for caspase 3 (a–d) or PCNA (e–h). Green color is for desmin in all panels. The lining epithelial cells are not visible because they are not immunoreactive for desmin. More cleaved caspase 3–immunostained nuclei of mesenchymal cells are evident in the lung tissue sections from a preterm lamb managed by HFNV and an FA136 fetal control lamb (b and d, respectively) compared with another preterm lamb managed by IMV and an FA132 fetal control lamb (a and c, respectively). The surrounding cytoplasm of many of the red-stained nuclei is desmin-positive (green). Some yellow immunostain is visible, presumably reflecting physical overlap, within the height of the tissue section, of part of the red cell nucleus and green cytoplasm. Conversely, fewer PCNA-immunostained nuclei of mesenchymal cells are evident in the lung tissue sections from a preterm lamb managed by HFNV and an FA136 fetal control lamb (f and h, respectively) compared with another preterm lamb managed by IMV and an FA132 fetal control lamb (e and g, respectively). DAS, distal airspace.

Figure 5. Double immunofluorescence localization, using lung tissue from preterm lambs ventilated for 3 days by IMV or HFNV, as well as fetal controls (FA132 and FA136). All of the panels are the same magnification; scale bar is 25 μm. Red color is for caspase 3 (a–d) or PCNA (e–h). Green color is for vimentin. The lining epithelial cells are not visible because they are not immunoreactive for desmin or vimentin. More cleaved caspase 3–immunostained nuclei of mesenchymal cells are evident in the lung tissue sections from a preterm lamb managed by HFNV and an FA136 fetal control lamb (b and d, respectively) compared with another preterm lamb managed by IMV and an FA132 fetal control lamb (a and c, respectively). The surrounding cytoplasm of many of the red-stained nuclei is vimentin-positive (green). Some yellow immunostain is visible, presumably reflecting physical overlap, within the height of the tissue section, of part of the red cell nucleus and green cytoplasm. Conversely, fewer PCNA-immunostained nuclei of mesenchymal cells are evident in the lung tissue sections from a preterm lamb managed by HFNV and an FA136 fetal control lamb (f and h, respectively) compared with another preterm lamb managed by IMV and an FA132 fetal control lamb (e and g, respectively). DAS, distal airspace.

Figure 6. Summary of morphometric measurements (mean $\pm$ SD) made in lung tissue sections from the groups of preterm lambs managed by IMV (open bars) or HFNV (solid bars) ($n = 8/\text{group}$), as well as gestation control lambs (shaded bars) ($n = 4/\text{group}$). Gestation control lambs were (1) fetuses delivered at the gestation age that the preterm lambs were delivered (FS), (2) fetuses delivered at the gestation age when the preterm lamb studies were ended (FE), and (3) term newborn (TN) lambs. Apoptotic index (a) is greater in, whereas proliferation index (b) is lower, for the preterm lambs that were managed by HFNV than by IMV. The results also show that apoptotic index (a) and proliferation index (b) for the HFNV group of preterm lambs were more comparable to the matching attributes of the FA136 gestation control group. By comparison, the results for the IMV group of preterm lambs were more comparable to those of the FA132 gestation control group. Statistical comparisons were by ANOVA and Fisher's PLSD *post hoc* test. Key for statistical differences ($P < 0.05$): *different from IMV; #different from FS; @different from TN.

pressure ventilation decreased the work of breathing (38), improved tidal volumes and minute ventilation, and lowered esophageal pressures when compared with nasal CPAP (39). Despite the effectiveness of gas exchange, with histologic and morphometric improvement of the lung, much remains to be learned about how gas exchange occurs with HFNV.

Apoptosis and proliferation play important roles in normal lung development. Apoptosis is evident among less than 1.0% of fetal lung cells (40, 41). In contrast, during the alveolarization stage of lung development in rats, which occurs postnatally, about 20% of interstitial fibroblasts undergo apoptosis (42–44). During normal ontogeny, attrition of fibroblasts reduces the thickness of the parenchyma (45, 46). Proliferation also participates in normal lung development, primarily among endothelial cells (47). Combined, those changes presumably optimize parenchymal structure for efficient gas exchange.

Dysregulated apoptosis and/or proliferation have been proposed as contributing to the structural aberrations associated with acute and chronic lung diseases, including neonatal respiratory distress syndrome and subsequent BPD. For example, epithelial cells lining the distal airspaces had more TUNEL staining (17, 18) and caspase-3 immunostaining (18) in infants who died with BPD compared with stillborn infants who died without lung injury. However, differences in apoptosis of mesenchymal cells were not identified. Apoptosis also has been generally described in the lung of mechanically ventilated preterm baboons (16). Sustained cell proliferation among surfactant B-positive distal airspace epithelial cells occurred in preterm baboons managed by conventional mechanical ventilation (48). Our results provide new insight by showing that HFNV results in more apoptosis, and less proliferation, of mesenchymal cells at 3 days of continuous ventilation compared with IMV. These differences likely contributed to the thinner

distal airspace walls in the preterm lambs that were managed by HFNV compared with IMV.

The differences in alveolarization that occurred in the 3-day experiments are striking and raise questions about how such differences occurred. Because all of the lungs were fixed at the same static transpulmonary pressure of 25 cm H₂O, the differences in alveolarization are unlikely attributed to inconsistent lung inflation fixation. Species differences in alveolar formation may have contributed to the results that we observed, because sheep have more advanced alveolar formation at term gestation (49–51) than humans, baboons, and mice (52–57). The gestation age at which we delivered the preterm lambs coincided with the sacular stage of lung development (~ 30 wk of gestation in humans). The alveolar stage in fetal lambs begins about a week later. Therefore, the developmental window during our study coincided with transformation of lung parenchymal architecture that, if arrested, would be obvious.

Differences in oxygen exposure and mechanical ventilator-associated injury are two etiologic factors in BPD (58) that may have contributed to the different outcomes. As shown in Table 3, the HFNV and IMV groups required the same FiO_2 on the first day of life (0.58 ± 0.28 and 0.71 ± 0.11 , respectively; not significant). During the second and third days of the 3-day study, FiO_2 was reduced in both groups to sustain the target PaO_2 . The average FiO_2 during the second and third days was approximately 0.37 in the HFNV group and approximately 0.52 in the IMV group ($P < 0.05$ for each day). Because even modest levels of hyperoxia are anti-proliferative in lung (59–61), the greater fractional concentration of oxygen required for the IMV group to maintain the target PaO_2 might have impacted the results.

Mechanical ventilator-associated lung injury is related to the magnitude and duration of volutrauma and atelectotrauma (62–64). Cyclic overdistension of ventilated areas and collapse of atelectatic areas exposes the lung's cells to mechanical stretch that distorts the cells and extracellular matrix, leading to alterations of stretch-responsive genes, alterations that have downstream consequences on expression of growth factors and inflammatory mediators (65–67). We have previously shown in this model that 3 days of IMV resulted in elevated transforming growth factor- α and - β_1 mRNA expression, as well as elevated tropoelastin, fibrillin-1, fibulin-5, and lysyl oxidase mRNA expression compared with unventilated fetal control lambs and/or term lambs that were managed by IMV (24). Although we did not measure those markers in the present study, the fact that both studies used preterm lambs that were ventilated for 3 days raises the possibility that mechanical stretch was different between the two ventilation modes. The underlying mechanotransduction mechanisms that may have affected lung phenotype in the IMV group versus the HFNV group have yet to be identified.

Another consequence of mechanical ventilator-associated lung injury is local inflammation. In a different preterm lamb model, short-term (2 h) application of conventional mechanical ventilator resulted in almost sevenfold more neutrophils in alveolar washes and about twofold more hydrogen peroxide elicited from the leukocytes obtained in the washes than preterm lambs that were intubated but managed by endotracheal bubble CPAP (10). When that study was repeated, and PaCO_2 was kept the same (50–55 mm Hg) between the conventional mechanical ventilator and bubble CPAP groups for 6 hours, pro-inflammatory mediators were increased similarly between the groups (68). Whether pro-inflammatory mediators were differentially expressed in the lungs of our preterm lambs remains to be determined.

Our study may shed some light on whether alveolar simplification associated with prolonged IMV in preterm lambs

Figure 7. Summary of immunoblot and quantitative real-time RT-PCR results (mean $\pm$ SD) for apoptosis (a, c, e) or proliferation (b, d, f) made in lung tissue homogenates from the repeated group of preterm lambs managed by IMV (open bars) or HFNV (solid bars), as well as gestation control lambs (shaded bars) ($n = 4/\text{group}$). Homogenates were made from frozen samples of peripheral whole-lung tissue that was devoid of visceral pleura and central airways/vessels/connective tissue. Gestation control lambs were (1) fetuses delivered at the gestation age that the preterm lambs were delivered (FS), (2) fetuses delivered at the gestation age when the preterm lamb studies were ended (FE), and (3) term newborn (TN) lambs. The band at the far right in each gel is the native protein (a and b). mRNA expression is normalized to the FS control group (gray line at 100% on the y-axis). Molecular indices of apoptosis (cleaved caspase 3 protein abundance [a], mRNA expression of p53 [c] and caspase 3 [e]); and proliferation (PCNA protein abundance [f], mRNA expression of TGF- β [d] and c-Myc [f]) for the HFNV group of preterm lambs were comparable to the matching results for either the FE or TN gestation control groups. Statistical comparisons were by ANOVA and Fisher's PLSD *post hoc* test. Key for statistical differences ($P < 0.05$): *different from IMV; #different from FS; @different from TN.

inhibits lung growth or results from destruction and loss of alveolar secondary septa. Comparing the structural results for the preterm lambs to those for the gestation control lambs shows that the IMV group of preterm lambs had saccular parenchymal architecture, with few alveolar secondary septa. That architecture was the same as the lungs of fetal lambs that were matched for gestation age when the preterm lambs were operatively delivered (FA132 control group). Likewise, the biochemical and molecular results from our study also indicate that apoptosis and proliferation in the IMV group of preterm lambs remained at the same level as that of the FA132 control group. Those results suggest arrest of alveolar secondary septation. Arrest could result from failure of alveolar secondary

septa to form and elongate, and/or from collapse of the alveolar secondary septa into the distal airspace walls as the walls became distended by mechanical ventilation. Either potential mechanism could be facilitated by alterations in the composition of the mesenchymal compartment associated with elevated expression of pro-inflammatory mediators and extracellular matrix components and enzymes (24). On the other hand, a potential mechanism for tissue destruction could be tissue stress failure due to overinflation of the lung (69). However, when we observed lung tissue sections histologically or ultrastructurally, evidence of broken alveolar walls or secondary septa was not seen. Based on the aforementioned evidence, we suggest that alveolar simplification associated with prolonged

IMV in preterm lambs may be due to lack of normal developmental progression during the 3-day ventilation period. By comparison, the HFNV group had greater alveolar complexity, with alveolar secondary septation that was intermediate between indices of alveolarization in fetal lambs that were matched for gestation age when the preterm lamb studies ended (FA136 control group) and in term newborn lambs. Thus, lung growth appeared to proceed during the 3-day ventilation period in the HFNV group.

Our study has limitations. One limitation is that the ventilation period was only one time point: 3 days. Thus, our study cannot address the important question of whether long-term ventilatory support by HFNV would reduce chronic lung disease of prematurity in our model. However, preterm baboons that were managed by nasal CPAP ventilation for 28 days had findings suggestive of ameliorated BPD (11). A potential limitation of our study is that the preterm lambs were exposed, antenatally, to a single dose of maternal dexamethasone 24 to 36 hours before operative delivery, whereas the control fetal and term lambs were not. Therefore, the possibility exists that the lung-maturing and anti-inflammatory effects of antenatal corticosteroids may have contributed to more alveolar complexity, regardless of ventilation mode, than if corticosteroids had not been administered. While those effects are possible, the preterm lambs that were managed by IMV for 3 days for the present study had lung architecture indistinguishable from other preterm lambs that our group managed by IMV for 3 days, without antenatal corticosteroids (24). Another limitation of our study is that the preterm lambs were resuscitated with IMV for 2 to 3 hours before weaning to HFNV. Lung injury may have been initiated during that brief interval (10, 68, 70), when peak inspiratory pressure was approximately 25 cm H₂O, tidal volume was approximately 5 ml/kg, and PEEP was 8 cm H₂O. However, such an effect would have obscured the demonstrated differences in outcome parameters detected between the HFNV group and IMV group, as well as between both groups of preterm lambs and the gestation age-matched, fetal control groups. Studies using intubated preterm lambs have shown that bubble CPAP results in lower expression of pro-inflammatory cytokines than IMV (10, 68, 70). Other potential confounding differences between the two groups of preterm lambs were amounts of sedatives used and nutrition delivered. The IMV group of preterm lambs received more pentobarbital (3–5 mg/kg/h, intravenously), to keep them comfortable, compared with the HFNV group (~1–2 mg/kg/h pentobarbital, intravenously), which permitted the latter group to breathe spontaneously. Both groups received the same dosage of buprenorphine (5 µg/kg/3h, intravenously). The greater amount of sedatives given to the IMV group may have altered cellular processes in the lung, as well as motility of and absorption by the intestine. In that regard, enteral feeding was different between the two groups of preterm lambs. Enteral feeding of ewe colostrum and maturing milk was less in the IMV group (~25 ml/kg/d) compared with the HFNV group (~45 ml/kg/d). The difference was caused by residual milk in the stomach of the IMV group of preterm lambs, which required skipping feedings. Regardless of enteral feeding, plasma glucose concentration was kept constant (60–80 g/dl) for both groups of preterm lambs by adjusting intravenous dextrose administration. The difference in enteral feeding may have contributed to differences in alveolarization between the two groups of preterm lambs (71); however, the effect probably was minimal because of the 3-day duration of the study.

In summary, this report demonstrates that the balance between apoptosis and proliferation of mesenchymal cells in the distal airspace walls is disrupted by prolonged conventional

mechanical ventilation. Future studies focusing on the signals that regulate this balance will be important in devising specific interventions.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors acknowledge the important contributions by Dr. Ronald Bloom, Dr. J. Ross Milley, and Dr. Robert M. Ward during these studies, as well as the expert technical assistance provided by Ms. Nancy B. Chandler of the Electron Microscopy Core Facility, at the University of Utah. B.R. and M.L. jointly worked on this project, which partially fulfilled their respective requirements for a master's degree (Brent Reyburn, Midwestern University, College of Health Sciences, Glendale, AZ) and undergraduate honor's thesis (Marlana Li, University of Utah, College of Science).

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