Prolonged mechanical ventilation with air induces apoptosis and causes failure of alveolar septation and angiogenesis in lungs of newborn mice

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Mokres LM, Parai K, Hilgendorff A, Ertsey R, Alvira CM, Rabinovitch M, Bland RD. Prolonged mechanical ventilation with air induces apoptosis and causes failure of alveolar septation and angiogenesis in lungs of newborn mice. Am J Physiol Lung Cell Mol Physiol 298: L23–L35, 2010. First published October 23, 2009; doi:10.1152/ajplung.00251.2009.—Defective lung septation and angiogenesis, quintessential features of neonatal chronic lung disease (CLD), typically result from lengthy exposure of developing lungs to mechanical ventilation (MV) and hyperoxia. Previous studies showed fewer alveoli and microvessels, with reduced VEGF and increased transforming growth factor-β (TGFβ) signaling, and excess, scattered elastin in lungs of premature infants and lambs with CLD vs. normal controls. MV of newborn mice with 40% O2 for 24 h yielded similar lung structural abnormalities linked to impaired VEGF signaling, dysregulated elastin production, and increased apoptosis. These studies could not determine the relative importance of cyclic stretch vs. hyperoxia in causing these lung growth abnormalities. We therefore studied the impact of MV for 24 h with air on alveolar septation (quantitative lung histology), angiogenesis [CD31 quantitative-immunohistochemistry (IHC), immunoblots], apoptosis [TdT-mediated dUTP nick end labeling (TUNEL), active caspase-3 assays], VEGF signaling [VEGF-A, VEGF receptor 1 (VEGF-R1), VEGF-R2 immunoblots], TGFβ activation [phosphorylated Smad2 (pSmad2) quantitative-IHC], and elastin production (tropoelastin immunoblots, quantitative image analysis of Hart’s stained sections) in lungs of 6-day-old mice. Compared with unventilated controls, MV caused a 3-fold increase in alveolar area, ~50% reduction in alveolar number and endothelial surface area, >5-fold increase in apoptosis, >50% decrease in lung VEGF-R2 protein, 4-fold increase of pSmad2 protein, and >50% increase in lung elastin, which was distributed throughout alveolar walls rather than at septal tips. This study is the first to show that prolonged MV of developing lungs, without associated hyperoxia, can inhibit alveolar septation and angiogenesis and increase apoptosis and lung elastin, findings that could reflect stretch-induced changes in VEGF and TGFβ signaling, as reported in CLD.

bronchopulmonary dysplasia; neonatal chronic lung disease; lung growth and development; alveolar and pulmonary capillary formation; vascular endothelial growth factor; transforming growth factor-β; mechanical stretch; elastin

RESPIRATORY FAILURE OFTEN complicates the postnatal course of premature infants whose lungs are incompletely developed. Life-saving treatment of such infants using assisted ventilation with O2-rich gas frequently leads to a chronic form of lung disease that was initially described as bronchopulmonary dysplasia (BPD; Ref. 54). The clinical and pathological features of this disease have changed in recent years owing to major advances in perinatal care, including routine use of antenatal glucocorticoid therapy when premature birth is anticipated, postnatal surfactant replacement, and improved respiratory and nutritional support. The salient pathological feature of this BPD variant, here defined as neonatal chronic lung disease (CLD), is an apparent arrest of alveolar septation (17, 29), which is often attributed to lengthy exposure of the developing lung to high concentrations of inspired O2 (13, 56, 70) and associated lung cell apoptosis (19, 27, 47). CLD, however, seldom if ever occurs without a history of mechanical ventilation (MV), and in many cases there has been only modest hyperoxia during development of CLD (7, 59).

Lungs of infants who have died with BPD show evidence of defective alveolar septation (17, 29) and capillary formation (8, 20). These findings were associated with reduced lung expression of VEGF and one of its receptors, fms-like tyrosine kinase receptor (Flt-1, also called VEGF-R1). Similar structural abnormalities of the lung parenchyma and microcirculation and associated changes in pulmonary expression of VEGF and its receptors have been documented in authentic animal models of BPD (2, 9, 12, 18, 42). Other reports have shown evidence of increased transforming growth factor-β (TGFβ) signaling in the lungs of infants with evolving CLD (31, 34, 37, 66) and in animal models of CLD induced by hyperoxia (3, 52) or prolonged MV (12).

Our group (12) previously reported decreased lung expression of VEGF-A and its receptor, VEGF-R2 (also known as fetal liver kinase-1, Flk-1, or kinase domain receptor, KDR) in preterm lambs with CLD after 3 wk of MV with O2-rich gas. Similar findings were noted in 4-day-old mice that received MV with 40% O2 for 24 h (10, 11). Compared with unventilated control pups, mice that had MV with O2-rich gas exhibited reduced alveolar septation and increased lung cell apoptosis that was associated with decreased lung expression of VEGF-A and VEGF-R2, genes that are known to affect the formation of alveoli and blood vessels in the developing lung (63, 69). These studies could not determine whether the changes in lung structure and VEGF signaling resulted from prolonged hyperoxia or cyclic stretch, or both, applied to the developing lung. In addition, because the process of alveolar formation normally begins at ~4 days of age in mice (6), the lungs of these 4-day-old pups had an insufficient number of alveoli to allow for a meaningful comparison between control pups and those that received MV. Therefore, in this study, we applied MV with air for 24 h in 6-day-old mice, in which there is greater progression of lung septation, thereby enabling assessment of alveolar number as well as alveolar size.

Our goals were to test the hypothesis that MV with air impairs alveolar septation and angiogenesis in lungs of newborn mice, that such effects might reflect increased lung cell apoptosis, and that these changes could be linked to reduced...
lung expression of VEGF-A and/or its receptors, VEGF-R1 and VEGF-R2. We discovered that MV of 6-day-old mice with air for 24 h, compared with unventilated controls, resulted in a ~50% decrease in alveolar number with near tripling of alveolar size, increased apoptosis, diminished distal lung expression of the endothelial cell marker CD31, a >4-fold increase in lung abundance of pSmad2 protein, and a >50% reduction in lung VEGF-R2 protein with no change in lung content of VEGF-A or VEGF-R1 protein. MV with air also increased lung elastin and shifted the distribution of elastic fibers from septal tips to alveolar walls. These findings suggest that lengthy and repetitive mechanical stretch of the developing lung, without associated hyperoxia, induces apoptosis leading to reduced alveolar septation and angiogenesis and scattered and increased elastin and that increased TGFβ and decreased VEGF signaling likely contribute to the structural changes observed in the lungs of newborn mice after MV for 24 h.

METHODS

Animal Experiments

Brief description of experimental design. We studied 3 groups of 5- to 6-day-old BALB/c mice that weighed 45 ± 0.7 g (means ± SD) after vaginal birth at term. Two groups (9–12 per group) had a tracheostomy under ketamine (~60 μg/g body wt) and xylazine (~12 μg/g body wt) anesthesia, as previously described (10, 11), followed by MV for 24 h with air delivered at either 60 or 180 breaths/min (bpm). The rationale for using one respiratory rate that was slower and one that was faster than the normal breathing rate of newborn mice (~110/min; Ref. 49) was to compare effects on the lung of different respiratory patterns featuring different tidal volumes and peak inflation pressures, which have been shown to yield blood pH (7.30 ± 0.12) and PCO2 (37 ± 11) values within the physiological range (11). Control pups spontaneously breathed air for 24 h. Details of experimental conditions during MV were similar to those previously reported for 4-day-old pups and included maintenance of a neutral thermal environment, treatment with ampicillin and gentamicin, repeated sedation as needed, and feedings of 10% glucose solution and milk formula via an orogastric tube every 2 h (11). At the end of each study, pups received pentobarbital, ~150 μg/g body wt, for lung resection and postmortem studies that included quantitative structural analysis and immunohistochemistry (IHC) of fixed lung sections and protein measurements using standard immunoblot techniques. All surgical and animal care procedures and experimental protocols were reviewed and approved by the Stanford University Institutional Animal Care and Use Committee.

Postmortem Studies

Processing of lungs for quantitative histology. The lungs were filled via the trachea with buffered 4% paraformaldehyde (PFA) solution for fixation at an inflation pressure of 20 cmH2O for 24 h at 4°C, as previously described (11). The fixed lungs were excised to measure their volume by fluid displacement (62), followed by isotropic uniform random (IUR) sectioning of paraffin-embedded lungs, from which 4-μm tissue sections were stained with hematoxylin and eosin for quantitative assessment of alveolar area using the Bioquant image analysis system and alveolar number [radial alveolar counts (RAC)] as previously described (21). Some lungs (4–5 per group) were instilled with IHC Zinc Fixative (cat. no. 51-7438KZ; BD Pharmingen, San Jose, CA) and processed by the same protocol used for the PFA fixation procedure. A detailed description of the IUR sampling procedure and morphometric analysis is included in the data supplement online at the AJP-Lung Cellular and Molecular Physiology web site.

RESULTS

Lung structural changes after MV with air for 24 h. Figure 1A shows representative sections of lung obtained from 6-day-old mice that received MV with air at 60 bpm (tidal volume ~8 μl/g body wt) or 180 bpm (tidal volume ~5 μl/g body wt) for 24 h compared with unventilated controls. Pups that received MV at either 60 or 180 bpm displayed enlarged, simplified distal air spaces without detectable inflammation or injury. Total lung volume did not differ significantly in pups that received MV compared with controls (Fig. 1A).

Morphometric analysis of lung tissue sections showed a two- to threefold increase in alveolar area and a 40–50% decrease in...
RAC in mice that received MV with air at either 60 or 180 bpm compared with controls (Fig. 1B). There were no significant differences in alveolar area or RAC between groups of mice that received MV at 60 bpm compared with 180 bpm. These findings indicate that regardless of differences in respiratory pattern, the lungs of mice that received MV with air for 24 h failed to undergo normal septation during prolonged cyclic stretch, resulting in larger and fewer alveoli.

**Lung cell apoptosis and proliferation.** Failed lung septation associated with MV may result from increased lung cell apoptosis or decreased lung cell proliferation, or both, in response to prolonged cyclic stretch or exposure to O2-rich gas. We (10) previously showed that MV of 4-day-old mice with 40% O2 for 24 h led to a threefold increase in lung cell apoptosis, as assessed by both TUNEL staining of lung tissue sections and by immunoblot measurement of active caspase-3 protein, without a significant change in lung cell proliferation, assessed by PCNA staining of lung tissue sections. To determine the specific impact of cyclic stretch on programmed cell death or proliferation of lung cells, measurements were made in lungs of 6-day-old mice that received MV with air for 24 h compared with unventilated control pups that breathed air for 24 h. We observed a fivefold increase in the number of TUNEL-stained cells (Fig. 2) with a corresponding increase in active caspase-3 protein in the lungs of pups exposed to MV (Fig. 3). MV had no significant effect on cell proliferation as assessed by PCNA staining of lung sections. These findings, which are similar to those previously reported for 4-day-old pups that received MV with 40% O2 for 24 h (10), suggest that cyclic stretch of the lung during development causes septal loss leading to defective alveolar formation.

**Lung abundance of VEGF-A and its receptors.** As VEGF signaling plays a key role in regulating the formation of alveoli and lung capillaries during development, we measured lung content of VEGF-A and its receptors, VEGF-R1 and VEGF-R2, in lungs of 6-day-old mice that received MV with air for 24 h at 60 or 180 bpm compared with unventilated controls. MV had no significant effect on the amount of VEGF-A or VEGF-R1 protein in lung but caused a 50% reduction in lung abundance of VEGF-R2 protein (Fig. 4). Quantitative image analysis of lung tissue sections stained with VEGF-R2 antibody confirmed reduced abundance of VEGF-R2 after MV with air for 24 h (Fig. 5).

**Reduced pulmonary endothelial surface area after MV with air for 24 h.** IHC for CD31 showed a significant decrease of endothelial surface area relative to the surface area of distal lung parenchyma after 24 h of MV compared with unventilated controls that spontaneously breathed air for 24 h (Fig. 6, A and B).
This finding was confirmed by immunoblot measurements of CD31 protein in control lungs compared with lungs from pups that received MV for 24 h (Fig. 6C). The apparent discrepancy between the ~50% reduction of CD31 determined by IHC and the ~20% decrease found by immunoblot analysis probably reflects a relatively greater impact of MV on distal lung microvascular endothelium (assessed by IHC) with little or no effect on endothelial cell loss in larger blood vessels within the lung (included in the immunoblot measurements).
Fig. 4. Immunoblots for VEGF-A (A) and its receptors, VEGF-R1 (B) and VEGF-R2 (C), proteins relative to β-actin protein in lung, showing that MV with air for 24 h at 60 or 180 bpm yielded no significant change in either VEGF-A or VEGF-R1 protein, whereas MV with air at either respiratory rate caused a ≥50% reduction in lung content of VEGF-R2 protein. *Significant difference compared with Control group, P < 0.05. Values are means and SD. n = 4–5 per group.
Increased pSmad2 stain in distal lung after MV with air for 24 h. To determine whether mechanical force applied to the lung during a critical stage of development might increase activation of TGFβ, we measured distal lung expression of pSmad2 protein in pups that received MV with air for 24 h compared with unventilated controls. Smad2 is a downstream effector molecule of TGFβ signaling that is phosphorylated when TGFβ becomes activated (44, 50). MV for 24 h led to a 4-fold increase in lung abundance of pSmad2 protein (Fig. 7, A and B), indicating that prolonged cyclic stretch with air increases TGFβ signaling in the lung.

Increased lung deposition and dispersion of elastin after MV with air for 24 h. Using quantitative image analysis of Hart’s stained tissue sections, coupled with immunoblot measurements of tropoelastin protein in lung homogenates, we found that MV with air for 24 h increased both soluble elastin (tropoelastin) protein and elastic fiber density in lung compared with unventilated controls (Fig. 8, A–D, respectively). Elastin protein was most evident at the tips of septal crests in unventilated control pups, whereas elastic fibers were strewn throughout the walls of distal air spaces in pups that received MV for 24 h.

DISCUSSION

Important Features of This Experimental Model

What’s new about this study? This study shows for the first time that prolonged MV of the developing lung with air, without associated hyperoxia, inhibits alveolar septation and angiogenesis and is associated with increased apoptosis, activation of TGFβ, and reduced expression of VEGF-R2, a critical determinant of normal lung development. These structural and molecular changes that occurred in newborn mice after 24 h of positive-pressure MV are similar to changes that have been described in newborn infants with CLD (8, 15, 28, 29, 43, 67) and in authentic animal models of this disease (2, 12, 18, 42).

We (11) previously showed that MV of 4-day-old mice with 40% O2 for 24 h reduces lung expression of genes and proteins, including VEGF-A and VEGF-R2, which regulate formation of alveoli and pulmonary capillaries, and induces structural changes indicative of impaired lung septation without causing significant inflammation. Defective lung growth in these newborn mice was associated with increased apoptosis and deposition of widely dispersed elastic fibers in the walls of distal air spaces (10), which are well-documented features of the lung pathology seen in human infants with CLD (27, 43, 65) and relevant animal models of CLD (12, 19, 57).

The experimental approach used in this study was designed to avoid the lung injury that is often associated with hyperoxia and high inflation pressures, in keeping with the strategy currently applied in most newborn intensive care units to treat tiny infants with respiratory failure. We therefore used small tidal volumes (4–8 ml/g body wt) and low airway pressures (peak 13–20 cmH2O, mean 4–6 cmH2O), without hyperoxia, to provide adequate respiratory gas exchange while minimizing the risk of lung injury.

There are, however, major differences between neonatal mice that receive MV after birth at term gestation compared with premature infants and experimental models of CLD that have been created in premature baboons and lambs. These include a more robust respiratory drive, a more competent surfactant system, and the need for a tracheostomy instead of an endotracheal tube to deliver MV in newborn mice. Despite these differences, MV with air for 24 h yielded similar changes in lung structure and abundance of key proteins that regulate formation of alveoli and pulmonary capillaries and thereby affect lung growth.
We initially thought that MV using a relatively slow respirator rate (60 bpm) and relatively large tidal volumes (∼8 ml/g body wt) would yield greater adverse effects on lung growth than the more rapid respirator rate (180 bpm) and smaller tidal volumes (∼5 ml/g body wt). Failure to see such a difference may reflect similar mean airway pressures (4–6 cmH₂O) observed when these two ventilation strategies were applied and the fact that neither pattern of MV caused apparent lung injury. Consistent with these results is the previous report of little or no difference in the severity of CLD observed in preterm lambs after 3 wk of MV at a respirator rate of 20 bpm and tidal volume of ∼15 m/kg compared with a rate of 60 bpm and tidal volume of ∼5 ml/kg (2, 9). Thus exposure of developing lungs to positive-pressure MV, regardless of the inflation pattern used to deliver it, is likely the major determinant of impaired lung septation and angiogenesis.

Although we consider prolonged cyclic stretch the likely cause of the molecular, cellular, and structural changes observed in the lungs after MV with air for 24 h, it is possible that other factors could have contributed to these findings. These include the presence of a plastic tube in the trachea, suboptimal nutrition and nonuniform lung inflation causing intrapulmonary shunting of blood, and possibly reduced oxygenation. Postmortem histological assessment of the lungs did not show evidence of inflammation or detectable differences in lung inflation between mice that received MV compared with unventilated controls, but we cannot exclude the possibility that instillation of fixative at a pressure of 20 cmH₂O could have obscured subtle inflammatory changes in lung parenchyma or modest regional differences in lung inflation. Although all pups received feedings of glucose-containing solution and milk formula, which prevented weight loss during MV, their caloric and protein intake was considerably less than in nursing mice although similar to the unventilated control pups that were isolated from the dam for all but 4 of the 24 h. Because of the minuscule size of the pups, we were unable to successfully apply pulse oximetry to assess arterial oxygenation, and venous admixture in terminal heart blood samples rendered unreliable our PO₂ measurements (43 ± 7 mmHg after MV with air for 24 h, 31 ± 4 mmHg in unventilated controls). Lacking definitive in vivo assessment of respiratory gas exchange, we cannot be certain that undetected differences in oxygenation between mice that received MV with air compared with those that breathed air without MV could have contributed to our results.

Reduced Lung Abundance of VEGF-R2 Induced by MV with Air

Several studies have demonstrated defective lung septation and emphysema in mice rendered deficient in VEGF, or where its receptor VEGF-R2 was blocked (24, 30, 36, 46), indicating that VEGF signaling plays an important role in forming and maintaining normal alveolar structure during lung development. Another study using gene microarray analysis linked decreased expression of VEGF-R2 with failed alveolar formation in lungs of neonatal mice that had been treated with dexamethasone, which inhibits alveolar septation and lung growth (16). Reduced lung expression of VEGF-R2 was observed in preterm lambs that acquired CLD after 3 wk of MV with O₂-rich gas (12). Reports that VEGF treatment prevented the adverse effects of prolonged hyperoxia on alveolar and lung capillary formation in newborn rats underscores the key role of VEGF signaling in regulating lung septation and angiogenesis during normal pulmonary development and during repair of neonatal lung injury (35, 64).
Because there was no decrease in lung mRNA expression of either VEGF-A or VEGF-R2 in newborn mice after 8 h of MV with air (11), we surmised that a longer period of MV with air would not affect lung content of either of these proteins nor would it likely affect alveolar formation. MV with air for 8 h, however, caused a significant increase in lung mRNA expression of both tropoelastin and lysyl oxidase, which could contribute to aberrant lung growth (10). Moreover, a recent report described increased apoptosis of cultured fetal rat lung epithelial cells exposed to 20% cyclic stretch with air for 24 h, suggesting that lengthy MV with air might impair lung septation and thereby reduce alveolar formation (38). These findings led us to test the hypothesis that a prolonged period of cyclic stretch with air alone might cause lung structural changes similar to those observed in newborn mice that received MV with 40% O2. The discovery that lung abundance of VEGF-R2 protein decreased by >50% after 24 h of MV with air, accompanied by a fivefold increase in lung cell apoptosis and a ∼50% reduction in distal lung expression of CD31, is strong evidence that mechanical force applied to the lung during development can disrupt VEGF signaling, abrogate alveolar septation and angiogenesis, and thereby inhibit lung growth.

A recent report showed that VEGF-R2 is expressed not only on endothelial cells, but also on epithelial cells in alveolar septa of newborn mice (1). By light microscopy, we were unable to distinguish between VEGF-R2 immunostaining of distal lung epithelium and vascular endothelium, but the observation that VEGF-R2 protein in lung, measured by immunoblot, decreased by >50% after MV for 24 h, whereas CD31, also measured by immunoblot, fell by ∼20% after MV for 24 h, indicates that VEGF-R2 protein abundance likely was reduced in both pulmonary epithelial and endothelial cells or that there was greater loss of epithelial than of endothelial cells after MV with air for 24 h. These findings are consistent with the evolving concept that VEGF signaling between pulmonary endothelial and epithelial cells plays a critical role in promoting normal growth and development of the mammalian lung (73).

**Impact of MV with Air on Lung Cell Apoptosis**

This study provides new evidence that prolonged cyclic stretch of the developing lung, in the absence of hyperoxia, can cause apoptosis without a compensatory increase in cell proliferation. Previous in vitro studies showed increased apoptosis of cultured fetal rat lung fibroblasts and epithelial cells that were subjected to mechanical stretch for up to 24 h (38, 61). The increased apoptosis seen in cultured fetal rat type II cells after exposure to 20% cyclic stretch for 24 h was attributed to release of the proinflammatory cytokine IL-8 and decreased production of the anti-inflammatory cytokine IL-10 (38). Inflammation, however, was not a notable feature of the lung histology seen in our 6-day-old mice after MV for 24 h with either air or 40% O2, nor was there evidence of cytokine release or inflammatory cells in the lungs of 4-day-old mice exposed to MV with 40% O2 for 24 h (11).

Other investigators reported increased apoptosis of adult rat type II lung epithelial cells that were exposed to cyclic stretch for up to 24 h (26). This group subsequently showed that inhibitors of angiotensin-converting enzyme protected the epithelial cells from stretch-induced apoptosis by a mechanism involving bradykinin-mediated stimulation of the phosphoinositid 3-OH kinase-Akt-Bcl-2/Bcl-xL pathway, which is known to regulate cell survival (25).

**Impact of MV with Air on Activation of TGFβ Signaling in Lung**

This study is the first to show that MV of newborn lungs with air for 24 h results in TGFβ activation, as assessed by increased
pulmonary expression of pSmad2. Previous studies demonstrated that overexpression of TGFβ1 may cause lung structural changes that mimic CLD (23, 68). TGFβ1 also has been shown to down-regulate VEGF-R2 expression (41, 48) and cause apoptosis of vascular endothelial cells (39). Although VEGF is well-recognized as a prosurvival factor for endothelial cells, a recent report offered compelling evidence that in the presence of TGFβ1, VEGF/VEGF-R2 signaling can activate p38 (MAPK) to cause endothelial cell apoptosis (22).

In a study performed with newborn rats, MV for up to 6 h with 40% O2 and a large tidal volume (25 ml/kg) caused activation of TGFβ signaling and increased lung expression of connective tissue growth factor (71). Our finding that MV with air delivered with modest tidal volumes (5–10 ml/kg) for 24 h
caused a >4-fold increase in lung abundance of pSmad2 protein indicates that prolonged cyclic stretch of the lung during development, without associated hyperoxia or excessive tidal volumes, can activate TGFβ, which, in turn, may contribute to the increased apoptosis and elastin deposition and the reduced VEGF-R2 expression observed in the lungs after MV for 24 h.

How does mechanical stretch affect signaling pathways, namely TGFβ and VEGF, which regulate lung growth and development? In vitro studies showed that cyclic stretch stimulates production of TGFβ1 by a protein kinase C-dependent pathway in alveolar epithelial cells (72). Similar effects were reported in vascular endothelial cells and cardiac myocytes subjected to cyclic stretch (33, 75). Application of cyclic stretch to cultured human hepatic stellate cells for up to 24 h showed a time-dependent increase of TGFβ mRNA and protein that was linked to activation of Rho small GTP-binding proteins (60), which control cell adhesion and cytoskeletal organization and play a key role in mechanotransduction in vascular smooth muscle cells (55). The importance of this signaling pathway in modulating the stretch response of lung epithelial cells is unknown. In a study that examined the role of TGFβ signaling in a hyperoxic mouse model of CLD, hyperoxia accentuated the impact of TGFβ on apoptosis of cultured type II lung epithelial cells (3). Taken together, these studies are consistent with the notion that cyclic lung stretch may cause activation of TGFβ, which, in turn, may induce apoptosis of epithelial and endothelial cells and thereby reduce VEGF-R2 expression in the lung during development, as seen in newborn mice after MV for 24 h.

TGFβ1 also has been shown to increase tropoelastin mRNA and soluble elastin protein content in cultured neonatal lung fibroblasts (45), which likely contributes to the increased abundance and redistribution of elastic fibers observed in the lungs of preterm lambs with CLD (12). Dysregulated activation and signaling of TGFβ has been linked to the failed alveolar septation and defective elastin assembly reported in fibrillin-1 null mice, in which pulmonary emphysema is a prominent feature (53). These studies underscore the importance of mechanical forces in activating TGFβ signaling and altering the elasticity of the extracellular matrix, which, in turn, can impact VEGF signaling to adversely affect alveolar septation and lung growth.

Impact of MV on Lung Elastin Abundance and Distribution

Elastin, a major component of the pulmonary extracellular matrix, plays a critical role in normal alveolar septation and angiogenesis. Proper regulation of elastin synthesis, assembly, and distribution within the lung is essential for normal postnatal pulmonary function. Several studies have shown an association between excess, disordered elastin accumulation and defective development of alveoli and microvessels in lungs of premature infants who have died with CLD (14, 28, 43, 65). In an authentic animal model of this disease, lengthy MV with O2-rich gas increased elastin synthesis and redistributed elastic fibers from septal tips to the walls of distal air spaces in the lungs of premature newborn sheep (12, 57).

Recently, we (10) reported that MV with 40% O2 for up to 24 h in newborn mice caused a twofold increase in lung abundance of tropoelastin, whereas lung content of proteins that are essential for elastin assembly was either unchanged (lysyl oxidase, fibrillin-1, and fibrillin-2) or decreased (fibrillin-5 and emilin-1). Quantitative image analysis of lung sections showed that elastic fiber density increased by ~50% after 24 h of MV with O2-rich gas with elastin distributed throughout the walls of distal air spaces rather than at septal tips, as seen in unventilated control lungs. These striking lung matrix changes were associated with a fourfold increase of serine elastase activity and a threefold increase of apoptosis in lung, supporting the notion that increased elastin synthesis, coupled with increased elastase activity and reduced lung abundance of proteins that regulate elastic fiber assembly, could explain altered lung elastin deposition and defective alveolar septation, as seen in CLD (10).

Our finding of increased, scattered lung elastin after MV for 24 h with air suggests that prolonged cyclic stretch, rather than modest hyperoxia, is the primary stimulus that promotes elastin production and redistribution. Our (10) previous observation that MV with air for 8 h did not boost serine elastase activity in the lungs of 4-day-old mice serves as evidence that the matrix remodeling and apoptosis seen after prolonged MV may occur independently of elastin degradation.

The specific way that abnormal elastin synthesis contributes to failed formation of alveoli and lung capillaries in CLD is unclear. There is, however, considerable evidence that cyclic stretch may induce tropoelastin gene expression in the developing lung, which, in turn, may yield increased matrix elastin (32, 51). A recent study showed that MV with air for 12 h inhibited secondary crest formation and caused a near doubling of elastic fiber density in lungs of very immature fetal lambs that were kept in utero with an intact placental circulation (4). Another recent report indicates that mechanical forces related to matrix elasticity can affect VEGF signaling in microvascular endothelial cells by modulating the activities of two antagonistic transcription factors, TFII-I and GATA-2 (40). These two genes are known to govern expression of VEGF-R2 and thereby regulate angiogenesis. These new discoveries highlight the importance of mechanical forces in altering the elasticity of the extracellular matrix, which, in turn, can impact VEGF signaling to adversely affect alveolar septation and lung growth.

Biological and Clinical Implications

This study shows for the first time that MV with air for 24 h inhibits formation of alveoli and small pulmonary blood vessels, alters elastin abundance and distribution, and induces apoptosis of endothelial and epithelial cells in lungs of newborn mice. These changes are similar to those observed after MV with 40% O2 for 24 h, as previously reported (10, 11). Defective VEGF signaling and activation of TGFβ signaling, which has been shown to reduce expression of VEGF-R2 in endothelial cells, likely contribute to the defective lung septation and angiogenesis observed after prolonged MV of the lungs during development. Mechanical stretch, with or without modest hyperoxia, appears to be the major stimulus for apoptosis leading to impaired alveolar septation and increased deposition and dispersion of lung elastin. Increased lung cell apoptosis and elastin deposition also could be linked to stretch-induced activation of TGFβ, which has been shown to induce apoptosis of endothelial cells (39, 58) and upregulate elastin
Mechanical ventilation with air during a critical stage of alveolar growth could have adverse effects on lung development in newborn mice. MV (prolonged cyclic stretch) of the lung during postnatal development activates TGFβ, which induces endothelial and epithelial cell apoptosis and impairs VEGF signaling (reduced expression of VEGF-R2). TGFβ also increases production and dispersion of elastin from lung myofibroblasts. These changes can result in defective alveolar and lung capillary formation, regarded as hallmarks of the pathology seen in chronic lung disease (“the new bronchopulmonary dysplasia”).

Figure 9 shows our working model depicting how lengthy MV during a critical stage of postnatal lung growth might lead to impaired alveolar septation and angiogenesis. Prolonged cyclic stretch of the developing lung results in activation of TGFβ, which stimulates tropoelastin release from lung myofibroblasts and induces apoptosis of endothelial and epithelial cells. These changes yield increased deposition and dispersion of elastin and reduced lung abundance of VEGF-R2, respectively. Lung structural abnormalities reflect diminished alveolar septation and capillary formation, which are quintessential features of the defective lung growth observed in CLD. Studies designed to judge the effects of inhibiting TGFβ signaling and/or upregulating lung expression of VEGF-R2 are needed to determine whether this model might account for the defective lung growth observed after prolonged MV of newborn mice.

Apart from its relevance to the pathogenesis of CLD, the finding that lengthy MV with air can interfere with normal lung growth may have important implications for term infants who require prolonged respiratory support in an intensive care setting. These include infants with acute encephalopathy, surgical conditions that impair normal breathing, and severe congenital heart defects. Such infants often are ventilator-dependent for several weeks, typically without need for supplemental O2. Although little is known about the long-term effects of positive-pressure MV on lung development in such infants, the results of this study suggest that even gentle MV with air during a critical stage of alveolar growth could have adverse effects on pulmonary structure and function later in life.

**DISCLOSURES**

No conflicts of interest are declared by the author(s).

**REFERENCES**


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MECHANICAL VENTILATION WITH AIR IMPAIRS LUNG GROWTH IN NEWBORN MICE


