Ventilation-induced Alterations in Lung Development

Veranderingen in long ontwikkeling veroorzaakt door ventilatie

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Chapter 1

General introduction

Mechanical ventilation is a lifesaving treatment in critically ill neonates. However, mechanical ventilation is also one of the most important risk factors (Table 1) of Bronchopulmonary dysplasia (BPD), the most common chronic lung disease in infancy with long-term pulmonary and neurological complications (1). Exposure of immature lungs to positive pressure ventilation results in oxidative stress and ventilator-induced lung injury. The resulting injury and inflammation lead to abnormal developmental and reparative processes in the lung.

This general introduction outlines the current knowledge regarding Bronchopulmonary dysplasia related to lung development, mechanisms of ventilator-induced lung injury, mechanosensing and mechanotransduction and pathways involved in the pathogenesis of BPD related to mechanical ventilation.

Table 1. Risk factors for development of BPD, related to gestational age in human.

Gestational age	Stage of lung development	Risk factors
24 - 32 weeks GA	Canalicular - Saccular	Genetic disposition, male gender, no antenatal steroids, fetal inflammation, chorioamnionitis
0 - 2 hrs postnatal	Saccular - Alveolar	High V_T, reactive oxygen species, no PEEP, no exogeneous surfactant
> 2 hrs	Alveolar arrest in development	Baro/volutrauma, reactive oxygen species, patent ductus arteriosus, fluid therapy, (mal)nutrition, infections

BRONCHOPULMONARY DYSPLASIA AND LUNG DEVELOPMENT

In the mid-1960's mechanical ventilation was introduced to manage premature infants with respiratory insufficiency because of respiratory distress syndrome (RDS). Soon, radiographic and pathologic abnormalities were observed, implicating barotrauma and oxygen toxicity as the primary causal factor of these abnormalities. Northway *et al*. were the first to describe this lung injury in preterm infants following mechanical ventilation. (2). They called it bronchopulmonary dysplasia (BPD). The infants ranged from 1474 to 3204 g birth weight and 30 to 39 weeks gestational age. Their chest radiographs revealed over-inflation and cystic emphysema and pulmonary histopathology consisted of emphysema, atelectasis, fibrosis, marked epithelial squamous metaplasia and smooth muscle hypertrophy in the airways and the pulmonary vasculature. These changes were associated with severe respiratory failure with airway obstruction, pulmonary hypertension, and cor pulmonale. The lung damage in these infants was attributed primarily to the use of aggressive mechanical ventilation and high inspired oxygen

concentrations. In 1969, Pusey *et al*. described diffuse interstitial fibroplasia associated with mechanical ventilation in newborns including patients without RDS. There was no clear relationship demonstrated between the use of high oxygen concentration and BPD, thus suggesting barotrauma as the primary cause (3).

Later advances in neonatal therapies, including exogenous surfactant, antenatal corticosteroids, newer modes of mechanical ventilation, improved nutritional management and better nursing techniques led to improved survival of infants born more immature than those described by Northway *et al.* and the histopathological features of BPD changed dramatically. Besides dysmorphic vasculature, the consistent lesion seen in this 'new BPD' is alveolar simplification and enlargement; it results from impairment in postnatal alveolarization in an extremely immature lung following premature birth (4-7). The extent of lung development between 24 and 26 weeks gestation and 30 and 32 weeks gestation is substantive; extensive vasculogenesis within the developing terminal saccules, followed by secondary crests formation along with interstitial extracellular matrix loss and remodeling. This tissue remodeling requires coordinate regulation of cell proliferation and apoptosis. Although alveoli are present in some infants at 32 weeks, they are not uniformly present until 36 weeks during the alveolar stage of development. Premature birth and initiation of pulmonary gas exchange interrupts this normal alveolar and distal vascular development.

Although the characteristics of BPD have changed, the incidence of BPD has remained the same (8, 9) or has increased and the disorder is no longer limited only to the most severely ill preterm infants (10, 11). BPD rates are slightly higher in very-low birth-weight infants and decrease incrementally with increased gestational weight; with >50% of infants less than 750 g, 15% of infants greater than 1000 g, and about 7% of infants greater than 1250 g (12, 13).

The pathogenesis of BPD is multi factorial (Fig. 1). Animal models have contributed greatly to the understanding of the etiologic agents of BPD. The main risk factors predisposing patients to BPD include prematurity, mechanical ventilation, oxygen therapy, infection and patent ductus arteriosus (Table 1). Invasive mechanical ventilation is one of the major contributors to the development of BPD in infants. There is currently no ideal ventilation strategy that minimizes the risks of pulmonary sequelae in premature infants.

Unfortunately mechanical ventilation is an inevitable intervention in the neonatal intensive care unit and thus BPD may not entirely avoidable. Understanding the pulmonary injury sequence is important to the realisation that some lung damage may be minimised by using lung protective strategies and to develop new therapies to minimise or prevent ventilator-induced lung injury in the premature infant.

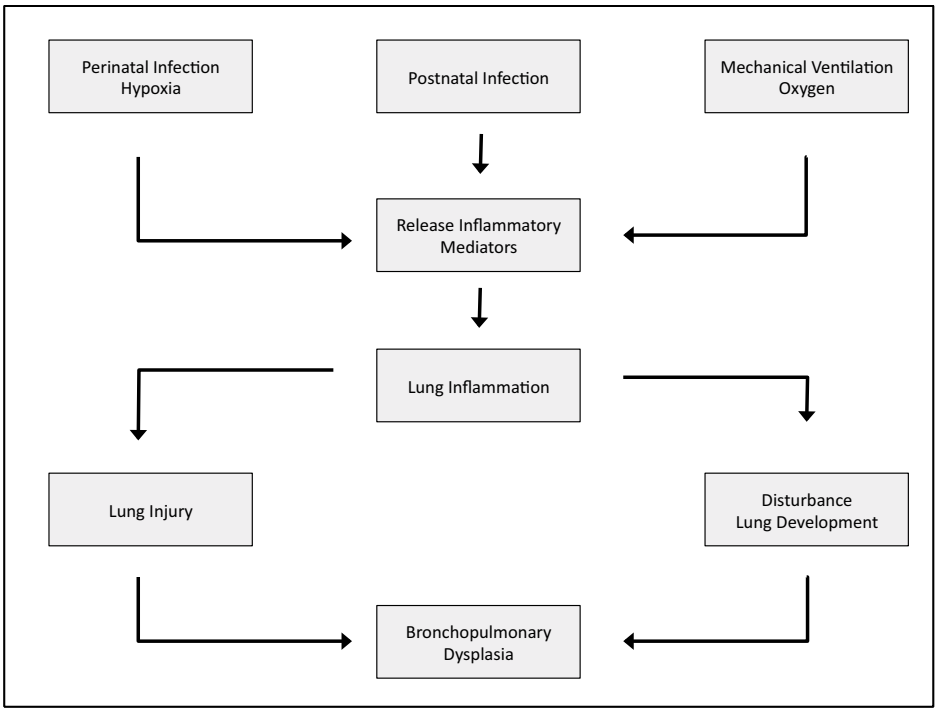

Figure 1. Concept of pathogenesis of BPD

MECHANISMS OF VENTILATOR-INDUCED LUNG INJURY

Barotrauma

Barotrauma is pressure–induced lung injury. Mechanical ventilation may cause pulmonary barotrauma through positive pressure. It can have a variety of clinical manifestations, including pneumothorax, pulmonary interstitial emphysema, pneumomediastinum and pneumopericardium. Barotrauma was suggested to be the most important factor in the pathogenesis of BPD until it was demonstrated that ventilation with high pressures without high volumes was not associated with increased lung oedema, in contrast to ventilation with low pressures and high volume (14). High pressure ventilation of premature rabbits did only result in increased microvascular permeability in the lung if chest expansion was not limited by a cast (15). Although these data indicate that volume distension of the lung rather than high peak pressures per se are more responsible for lung injury (16), barotrauma is still considered to be an important risk factor for the development of BPD (17).

Volutrauma

Volutrauma is injury caused by excessive tidal volume delivery. Ventilation with high tidal volumes leads to regional overdistension of alveoli and airways. In newborn animals this results in damage to the pulmonary capillary endothelium, alveolar and airway epithelium and the basement membranes (18, 19). The result is a leakage of fluid, protein and blood into the airways, alveoli and the lung interstitium, interfering with lung mechanics, inhibiting surfactant function and promoting lung inflammation (20-22). Adding positive end-expiratory pressure (PEEP) diminishes the severity of tissue injury (20, 23).

Atelectotrauma

Atelectotrauma is injury caused by repeated opening and closing of lung units. Cyclic opening and collapse of alveoli increases stretch and shear forces, resulting in lung injury and surfactant dysfunction. In an *ex vivo* rat lung model cyclic opening and closing from zero end-expiratory pressure (ZEEP) led to increased cytokine levels in the broncho-alveolar lavage fluid (24). Lung compliance decreased dramatically after ventilating rat lungs with a PEEP below the lower inflection point of the inspiratory pressure-volume curve compared to ventilation with PEEP levels above the inflection point. Histological analysis showed epithelial denudation and/or necrotic debris in the airways (25). PEEP above the lower inflection point minimized the influx of activated neutrophils into the lung of a surfactant-depleted piglet model (26). Possibly only the deflation limb of the pressure-volume curve provides the right information about the level of PEEP to prevent collapse of the alveoli during expiration (27, 28).

Biotrauma

Biotrauma is caused by the release of pro-inflammatory cytokines secondary to epithelial injury in the lung which contributes to a systemic inflammatory cascade of both pro-inflammatory and anti-inflammatory mediators (29). Especially high tidal volume ventilation and high inspiratory pressures can trigger an inflammatory response (30, 31). But even ventilation with low tidal volumes caused lung inflammation with impaired alveolarization and capillary development in a premature baboon model (32). Inflammation is a response to injury, resulting in recruitment of blood leucocytes, activation of tissue macrophages and production of inflammatory mediators. The inflammatory mediators are released by bronchial, bronchiolar and alveolar cells and by alveolar macrophages and neutrophils (33, 34). The inflammatory response may lead to cell regeneration and healing or progression of the inflammatory response, leading to a systemic inflammation (35). The immune response depends on the balance between pro-inflammatory, like tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL8, and anti-inflammatory cytokines, like IL-10 and IL-13 (36). TNF-α and IL-1β cause up-

regulation of vascular adhesion molecules, resulting in adhering of blood neutrophils to endothelial cells, after which they extravasate (37). Especially CXC chemokines and IL-8 activate and attract neutrophils into interstitial and alveolar spaces of the lung (38). This activation and attraction of leukocytes may play a very important in the pathogenesis of BPD (39, 40). Inhibition of neutrophil influx into the lung increased alveolar formation in a BPD model using newborn rats (40). NF-κB plays a central role in the generation of the pro-inflammatory mediators. Anti-inflammatory IL-10 and IL-13 inhibit NF-κB activation (41). Elevated concentrations of IL-1β, IL-6 and IL-8 in amniotic fluid and bronchoalveolar lavage fluid (BALF) have been associated with BPD (42, 43).

MECHANOSENSING AND MECHANOTRANSDUCTION

Mechanosensing

Lung cells sense and integrate information from mechanical distortion. Detecting the mechanical stimuli on the cell and converting it to into a biological signal is called mechanosensing (44). Mechanosensing is essential for the development and physiology of the lung cell (45), but is also suggested to play a role in the development of ventilator-induced lung injury (31). Integrins, the cytoskeleton, stretch-activated ion channels, growth factor receptors and cell-cell adhesion molecules are likely all involved in mechanosensing (44, 46). Integrins are transmembrane proteins, which anchor the cell on the basement membrane and extracellular matrix. They are in close contact with other proteins like cytoskeletal proteins and tyrosine kinases which are important for anchoring the cytoskeleton to the plasma membrane (47). The cytoskeleton itself may also sense cellular deformation (48). Mechanical stretch can affect the permeability of the plasma membrane to various ions. These changes in the concentration of specific ions in the cell lead to conversion of the physical stimulus into an electrical or chemical signal (44). The role of these channels in the pathogenesis of ventilator-induced lung injury is still unknown. Studies suggest that vascular endothelial growth factor (VEGF) receptor-2, receptor tyrosine kinases and platelet endothelial cell adhesion molecules-1 (PECAM-1) are involved in sensing mechanical stress in endothelial cells (49, 50).

Mechanotransduction

Mechanotransduction is the conversion of mechanical stimuli into biochemical and biomolecular signals (46). It has become evident that the mitogen-activated protein kinase (MAPK) has a key position in the process of mechanotransduction (51-53). After cellular deformation is sensed by the mechanosensor apparatus, the MAPK pathway is activated. As a result the mechanical signal is transduced to the nucleus and gene transcription is stimulated by activation of transcription factors (54). Most described

MAPK members are the extracellular signal-regulated kinase-1 and -2 (ERK1/2), the p38 MAPKs and the c-jun-N-terminal kinases (JNKs) (55). The MAPK pathway can be activated by a variety of extracellular signals including growth factors, cytokines, chemical and physical stress (54). The MAPK signaling cascade controls essential cellular functions, such as cell proliferation, programmed cell death and cell motility (54). Important transcription factor increased by cyclic stretch are IL-8, IL-6 and Egr-1 (53, 56, 57), which are associated with ventilator-induced lung injury (42, 43, 58). Another pathway activated by stretch is the NF-κB pathway (52, 53, 59).

HOW MECHANICAL VENTILATION CAN INFLUENCE LUNG DEVELOPMENT

Proliferation

Normal lung development requires coordinated proliferation and differentiation of epithelial and mesenchymal cells. Cell proliferation and differentiation are controlled by cell-cycle progression, a highly organized and tightly regulated process. Because proliferating and non-proliferating epithelial or mesenchymal cells lay beside each other, both growth stimulatory and inhibitory pathways are likely to be activated at the same time.

Disruption of the cell cycle regulation can result in developmental abnormalities. The cell cycle of the standard eukaryotic cell is divided into four non-overlapping phases (Fig. 2), with DNA synthesis during the S phase and mitosis during the M phase. These two phases are separated by G_1 and G_2 phases during which mRNAs and proteins accumulate continuously. In G_1 phase the cell is preparing for DNA synthesis (60, 61). In the subsequent S phase, DNA duplication occurs. Before cells undergo mitosis they continue in the G_2 phase with cell growth, preparing for cell division in M phase. Cell which are in G_0 phase are not actively cycling (60). Each phase of the cell cycle contains checkpoints that allow the arrest of the cell cycle progression and activation of repair mechanisms or even the apoptotic cascade, leading to cell death. After passing these checkpoints cells go irreversible into the next phase (62). The key regulatory proteins, which allow the transition from one cell cycle phase to another, are called cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases, which are activated at specific points during cell cycle (60, 63). Positive regulation of CDK activity occurs through association with a cyclin and by phosphorylation via a CDK-activating kinase (62). Cyclins are produced at each of these phases and form a complex with their CDK partner. The level of activating cyclins in different stages of the cell cycle differ, whereas CDK protein levels remain constant (63). During the G_0 to S phase transition, cyclins D1, D2, D3 and C get activated. Cyclins D1, D2 and D3 bind to CDK4 and CDK6. These cyclins promote entry into the G_1 phase. Cyclin C binds to

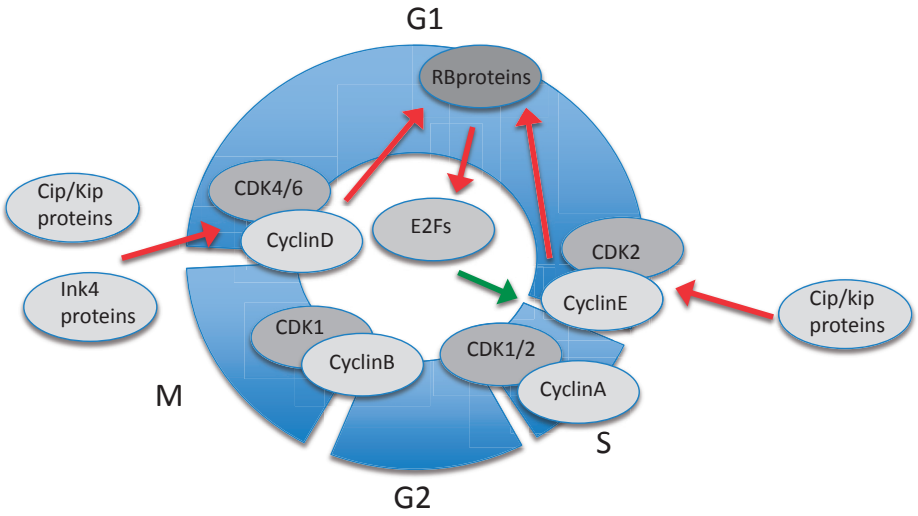

Figure 2. How different proteins influence cell cycle regulation. The cell cycle is divided in four phases: the G1, S, G2 and M phase. Cyclin-dependent kinases (CDKs) form subunits with cyclins, promoting entry into the next phase by phosphorylating and inactivating retinoblastoma protein (RB) (red arrow). This results in expression of E2F inducible genes, leading to entry into the S-phase (green arrow). Cip/Kip ($p21^{WAF1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) and Ink4 proteins ($p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$) inhibit the formation of cyclin-CDK complexes (red arrows).

CDK8. Cyclin E activates CDK2 and is associated with transition during G1 to S phase. Cyclin A gets activated during the S phase transition and binds to CDK1 and CDK2. B type cyclins are present during G_2 and M phases and are associated with CDK1 (64). G and T type cyclins are associated with CDK5 and CDK9, respectively (65). Active CDK4/6-cyclin D complexes phosphorylate retinoblastoma susceptibility protein (RB) (66, 67). RB reacts with a wide array of proteins that are mostly involved in transcription control. Among these proteins transcription factors E2Fs are very important. They are central regulators of cell cycle gene expression, and directly regulate the expression of genes involved in DNA replication, DNA repair, and G_2/M progression. Sufficient phosphorylation of RB proteins inactivates its transcriptional repressor function, thus allowing expression of E2F target genes, whose activity is essential for entry into S phase (68). CDK's are also tightly regulated by inhibitory phosphorylation, mediated by the kinase Wee1 and MYTC1 (62) and by two families of inhibitors: Ink4 proteins and the Cip/Kip family. Ink4 proteins include $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$. Ink4 proteins can inhibit cyclin D-associated kinase activity via binding to the CDK4 subunit (69, 70). Cip/Kip family proteins include $p21^{WAF1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. Cip/Kip proteins bind to and inhibit cyclin-CDK complexes. Preferentially they act on cyclin E-CDK2 complexes *in vivo* (71). Over expression of these two families of inhibitors blocks cell proliferation (72-76).

Fetal lung cell growth

Fetal lung growth is dependent on several mechanical forces, including sufficient amniotic fluid and normal fetal breathing movements (FBM) (77, 78). The fetal lung actively secretes fluid into the lung lumen, creating a constant transpulmonary pressure in the potential airway and air spaces (79). The volume of this lung fluid is dependent on the diaphragmatic contractions associated with FBM (80). In the first trimester of intra-uterine life the fetus makes episodic breathing movements, which increase in frequency to 30% of time by birth, (81). Abolishing FBM by sectioning the upper cervical cord or phrenic nerves resulted in reduced lung growth and maturation (82, 83). The critical stimulatory role of mechanical stretch on lung growth, secondary to increased intratracheal pressure has extensively been investigated in tracheal occlusion (TO) studies (84-87). Tracheal occlusion demonstrated a significant influence on proliferation of alveolar type II cells (85, 87). Alveolar Type II cells are of critical clinical importance. They produce and secrete surfactant and are progenitors of the gas-exchanging type I cells (88). This stretch-related proliferation of alveolar type II cells is dependent on timing and duration of stretch. In a fetal rabbit model, a significant increase in alveolar type II cells proliferation was noted after TO in the late pseudoglandular stage, whereas TO in the late canalicular stage had no significant effect (87). Fetal sheep, exposed to 2, 4 or 10 days of TO in the alveolar stage of lung development, showed an increase in the number of alveolar type II cells only during days 2 to 4 (85). Fetal sheep exposed to TO in the early canalicular stage of lung development showed increased lung growth, but a decrease in alveolar type II cells (86). After 10 days of TO this decrease in alveolar type II cells coincides with an increase in alveolar type I cells, suggesting induced differentiation of alveolar type II cells in type I cells by sustained lung expansion (89). This gestation-dependent proliferative response of fetal lung cells to stretch was also demonstrated *in vitro*. Intermittent stretch (simulating normal FBM) for 48 hours of fetal rat lung cells (epithelial cells and fibroblasts) stimulated lung cell proliferation at 19 days of gestation (90). In contrast, intermittent mechanical stretch for 24 hours of fibroblasts isolated from fetal rat lungs at 19 days of gestation decreased proliferation by 42% compared to unstretched controls (91). Only stretching of fetal lung cells isolated on day 19 (canalicular stage of lung development) of gestation showed these effects. Stretching of cells isolated on day 18 and 20 did not alter the number of proliferating cells. These data from *in vivo* and in *vitro* studies indicate that mechanical stretch is essential for normal lung development and that responsiveness of lung cells to mechanical stretch is dependent on stage of lung development and duration of stretch. Very little is known how mechanical stretch influences the cell-cycle regulation of lung cells with regard to the proliferation The involvement of the cyclin system has mainly been studied in oxidative stress models. Hyperoxia induced the expression of $p21^{WAF1/Cip1}$ in newborn mice (92) and in lung epithelial cells (93) after at least 48 hours. Because

hyperoxia induces a BPD-like pathology (86, 94), this induction of $p21^{WAF1/Cip1}$ may be responsible for disrupting coordinated cell proliferation and alveolarization. Indeed, in a premature born baboon model of BPD, baboons showed a threefold increase in $p21^{WAF1/Cip1}$ expression. In contrast to normal lung development, during disease progression the proportion of proliferating type II cells increased and there was a temporal shift in $p21^{WAF1/Cip1}$ expressing cells from type II cells at day 4-6 to mesenchymal cell at 14-21 days. The animals received ventilator support and oxygen as needed to achieve normal blood-gas measurements (95). In the same model increased expression of Cyclin D1, Cyclin E and Cdk4 was demonstrated at day 6-14, while that of Cyclin A and RB protein was decreased (96). Since the animals had lung injury, it is possible that ongoing lung repair demands an increased proliferation of cells at this time point. A possible candidate for the increased expression of $p21^{WAF1/Cip1}$ is transforming growth factor-β (TGF-β). This cytokine is induced by hyperoxia (97) and can increase the transcription of $p21^{WAF1/Cip1}$(98).

Apoptosis

Cells can die in three different ways, by programmed cell death, oncosis or autophagy (99). Programmed cell death is a process of controlled cell death and is important in the development and remodeling of tissues that occur during the normal repair process (91, 100). Apoptosis is only one of several types of programmed cell death (101). Apoptotic cells are characterized by cytoplasmic, nuclear shrinkage and the formation of apoptotic bodies. The cellular membrane of apoptotic cells remain intact (102). The result is the complete elimination of cellular debris without induction of an inflammatory response (103, 104). Apoptosis is genetically determined during the coarse of development, but can also be triggered by external stimuli, such as inflammation and intrinsic stimuli, resulting from alteration of cellular function and metabolism. During apoptosis proteases are activated. Of these proteases, caspases are the most extensively studied. The human genome encodes 13 distinct caspases of which seven have been suggested to participate in apoptosis as initiator (caspase-2, -8, -9 and 10) or effector (caspase-3, -6 and 7) (105). Caspases can be activated by either the extrinsic pathway or by the intrinsic pathway (106, 107). The extrinsic pathway is triggered by the ligation of cell surface death receptors and their ligands, such as tumor necrosis factor (TNF), Fas ligand (FasL) and TNF-related apoptosis-inducing ligand. When the Fas receptor binds its ligand, this recognition event is translated into intracellular signals that eventually lead to caspase-3 activation, through caspase-8 activation (106). Activated caspase-3 further triggers enzymes responsible for apoptosis, resulting in nuclear condensation and fragmentation (105, 108). The intrinsic pathway is characterized by changes in mitochondrial outer membrane integrity, which is regulated by Bcl-2 family proteins. In this family both anti-apoptotic and pro-apoptotic proteins are recognized (108). The

balance between these two groups of proteins determines the integrity of the mitochondrial outer membrane. Permeabilization of the mitochondrial outer membrane will lead to release of mitochondrial molecules, of which cytochrome *c* is the most critical factor in the intrinsic pathway. When cytochrome *c* is released in the cytosol a cascade of different caspases will be activated. Finally, caspase-3 will be activated, leading to cell death. In certain conditions the extrinsic and intrinsic pathway are interlinked by caspase-8 (108). Besides caspase activation as executioner of programmed cell death, there is increasing evidence of the existence of caspase-independent pathways leading to apoptosis (109, 110).

Oncosis is characterized by a progressive loss of cytoplasmatic membrane integrity, cytoplasmatic swelling and both lysosomal and mitochondrial swelling and disruption, leading to cellular fragmentation and leakage of lysosomal and granular contents into the surrounding extracellular space (111). Calpains, a family of Ca^{2+}-activated neutral cysteine proteases, are considered to play a role in this type of cell death (112). They are activated by the increased cytoplasmatic concentration of free calcium. In contrast to apoptosis, oncosis is often associated with inflammation in the adjacent tissues (99). Usually oncosis is associated with ischemia and sudden fall in tissue oxygenation. Bacterial exotoxins and mechanical stretch also may cause oncosis (113).

Autophagy is a process in which cells digest their own organelles and macromolecules to generate energy and metabolites to survive during a period of starvation or when it is deprived of growth factors. Cells that digest all available substrates will die eventually. This type of cell death is characterized by vacuolation and degradation of cytoplasmatic components (114). Autophagy is an adaptive response to sublethal stress, such as nutrient limitation, heat, oxidative stress and/or the accumulation of damaged or excess organelles and abnormal cellular components (115, 116). It also has a role in the suppression of tumor growth, deletion of toxic misfolded proteins, elimination of intracellular microorganisms and antigen presentation (116). Malfunction of autophagy contributes to a variety of diseases including cancer, neurodegeneration, cardiovascular diseases and microbe infections (114). However, whether autophagy is involved in ventilator-induced lung injury remains to be demonstrated.

Apoptosis and stretch of fetal lung cells

During lung development and maturation alveolar type II cells undergo apoptosis. Three peaks of apoptosis have been described: during transition from the canalicular to saccular stages of lung development, during birth and during the third postnatal week (100, 117-119). The last peak coincides with alveolar septal formation. Peaks of apoptosis are preceded by peaks in proliferation of lung cells, indicating the significance of apoptosis during lung development by removal of excess lung cells (117-119). Apoptosis is also responsible for restoration of the alveolar epithelium after lung injury (120). The role of

stretch-induced apoptosis in lung development was first demonstrated in a fetal rabbits after TO. Ligation of the trachea caused a depletion of alveolar type II cells. Different *in vitro* studies have demonstrated that cyclic stretch of day 19 fetal rat type II cells and fibroblasts for 24 hours increased apoptosis (91, 121). These cells were exposed to 5% elongation to mimic FMB. These data demonstrate the important role of physiological stretch induced-apoptosis for lung remodeling.

However the mechanisms by which excessive stretch of fetal lung cells contributes to the development of BPD are not well defined. Exposure of fetal lung cells to 20% elongation to simulate ventilation-induced lung injury increased the number of apoptotic cells compared to cells exposed to 5% elongation (121). A relation between increased apoptosis and reduced alveolar formation was recently demonstrated (122). Mechanical ventilation for 24 hours of newborn mice led to a 5-fold increase of apoptotic lung cells. *In utero* ventilation of fetal sheep for 6 hours increased apoptosis of mainly interstitial cells (123). The increases in apoptosis coincided in both studies with an significant reduction in alveolarization. Only one study has reported the effect of ventilation on apoptosis in infants with BPD. In lung sections from ventilated preterm infants delivered at 22-36 weeks the number of apoptotic epithelial cells increased significantly compared to still born foetuses (104). Besides ventilation, these infants were also treated with additional oxygen.

That apoptosis of fetal lung cells is triggered by mechanical stretch is now widely recognized. However, which signaling pathways are involved remain to be elucidated. Stretch induces the activation of caspases (91, 122). It has been suggested that activation of the Fas/FasL system is pivotal in activating the caspases (124). Sustained lung expansion in fetal rabbits resulted in a time-specific increase of FasL protein in alveolar type II cells, synchronous with alveolar type II cell apoptosis (125). This time-specific activation of FasL protein was also demonstrated in fetal lung fibroblasts (91). Stretching of day 19 fetal rat fibroblasts induced apoptosis without detection of FasL. Obviously the Fas/FasL system is only activated by mechanical stretch during the late-gestational stage of lung development, leading to alveolar type II cell apoptosis. This is plausible considering the time-specific up-regulation of FasL mRNA and protein during lung development (126). Thus, signaling pathways independent of FasL must be involved in stretch-induced apoptosis of alveolar type II cells during earlier stages of lung development and apoptosis of lung fibroblasts. TNF-α and its receptors represent another major extrinsic cell death pathway. Mechanical ventilation up-regulates TNF-α (127). But if this pathway is important in stretch-induced apoptosis besides FasL is not known. Other up-regulated pro-inflammatory cytokines/chemokines by mechanical ventilation are IL-1β, IL-6, IL-8, and TGF-β (121, 128, 129). Some of these inflammatory mediators have also pro-apoptotic properties. The role of TGF-β in the pathogenesis of BPD has more extensively been studied. Exposure of alveolar type II cells to hyperoxia increased

the sensitivity of these cells to TGFβ-induced apoptosis (130). Prolonged ventilation of newborn mice increased Smad2 protein, indicating increased TGF-β signaling (122). Smad2 is a downstream effector molecule of TGF-β signaling (131). TGF-β induces apoptosis by enhancing the Fas/FasL system via caspase-3 activation, but also via down regulation of $p21^{Cip1/Waf1}$ expression (124, 132). Expression of $p21^{Cip1/Waf1}$ causes not only cell cycle inhibition but also suppresses apoptosis (133), which makes the role of $p21^{Cip1/Waf1}$ in the pathogenesis of BPD complicated.

The extra cellular matrix

The extracellular matrix (ECM) plays an important role in lung development and the biomechanical behaviour of the lung. The macro molecules that constitute the ECM are: (1) fibrous proteins (collagen and elastin); (2) structural or adhesive proteins; and (3) proteoglycans and glycosaminoglycans (134). Elastic fibers are required for the vascular, conducting airway and terminal airspace compartments of the lung. Together with interstitial collagen, they form an elastic architecture that can undergo repeated deformation and recoil. For this property the orientation, thickness and length of the elastin fibers are important (135).

Initially, elastin is produced during the pseudoglandular stage of lung development (136). Alveolar elastin is produced primarily by α-smooth muscle actin-expressing cells of the alveolar wall (137). Elastogenesis is an essential step to the process of alveolar septation (138). Formation of new alveoli during the first phase of alveolarization starts with the development of new septa from immature preexisting septa. Elastin is organized into elastic fibers, which are composed of elastin and microfibrils. The microfibrils act as a scaffold on which elastin is deposited. Elastin is synthesized as tropoelastin. Fibulin5 and lysyl oxidase (LOX) play key roles during the assembly of tropoelastin into elastin fibers. Fibulin5 potently induces elastic fiber assembly and maturation, by alignment of tropoelastin on microfibrils and facilitating cross-linking of tropoelastin by lysyl oxidase (139, 140). Fibulin5 and elastin null mice exhibit abnormal alveolar development (138, 141). Lungs of LOX null mice display impaired development of distal and proximal airways with dilatation of the alveolar walls, sacs and primitive alveolar structures (141).

Extracellular matrix glycoprotein tenascin-C (TN-C) plays a morpho-regulatory role during development and tissue remodeling. It is expressed in the human lung during all developmental periods (142). Fetal lungs of TN-C deficient mice have enlarged airspaces (143). Antiserum to TN-C inhibited branching morphogenesis of fetal rat lung explants (144). Once the lung has developed, the expression of TN-C is absent or very low, but reappears under pathological conditions caused by infections, inflammation or during tumorigenesis (145). The expression of TN-C is regulated by different growth factors, including TGF-β1 (145, 146).

Blood vessels develop at the same time as the airways. Two processes of vascular growth have been described: angiogenesis and vasculogenesis. Angiogenesis is abluminal sprouting of new vessels from preexisting ones. This process concerns the formation of the large pulmonary vessels. The pulmonary capillary bed is formed by vasculogenesis. Vasculogenesis is the de-novo formation of vessels from precursor cells (angioblasts and endothelial precursors) in the mesenchyme. The airway acts as a template for the pulmonary vessels during this process (147). Hence, it is not surprising that different models of lung hypoplasia have shown that disrupted alveolarization is associated with abnormalities of the pulmonary circulation (148-150). The same observation has been made in diseases like BPD and congenital diaphragmatic hernia (151-154). Growth factor signaling, especially that of VEGF and PDGF, is important for regulating both the formation of alveoli and blood vessels. Inhibition of VEGF signaling using a receptor blocker decreased alveolarization in neonatal rats and mice (155, 156), while VEGF treatment enhances alveolarization (157, 158). The importance of PDGF-A in postnatal lung development has been demonstrated in PDGF-A deficient mice. These mice acquire pulmonary emphysema from failed alveolarization (159, 160). PDGF-α receptor signaling has been shown to contribute to the growth of pulmonary myofibroblasts, which play an important role in septal formation (161). Generally it is presumed that disruption of alveolarization will cause failure of lung vascular growth and that distruption of normal angiogenesis can also lead to impaired alveolarization. In which sequence mechanical ventilation interferes with alveolarization and vascularisation is yet unclear.

The extra cellular matrix and stretch

Mechanical forces have been shown to induce expression of tropoelastin *in vitro* (162) and *in vivo (163)*. The increase in tropoelastin mRNA expression coincided with an increase in elastin deposition, predominantly at the tips of secondary septal crests, and an increase in alveolar numbers (163). Sustained reduction led to decreased tropoelastin expression with disorderly elastin deposition in the alveolar walls with less secondary septal crests. A decrease in lung tropoelastin mRNA expression and lung elastin was also demonstrated in hypoplastic fetal rat and human lung (164, 165). This indicates that non-injurious stretch of the fetal lung is important for correct deposition of elastin and for alveolar formation. Prolonged ventilation with room air of 2-4 days old mice and fetal sheep resulted in increased, dysregulated elastic fiber deposition (122, 123). Prolonged ventilation with 40% oxygen did decrease the expression of fibulin5 and emilin1, while the expression of other genes regulating elastic fiber assembly was not changed (166).

Cyclic mechanical strain of human airway smooth muscle cells has been shown to increase TN-C expression (167). Furthermore, TN-C expression is down-regulated in

murine model of pulmonary hypoplasia with co-existent diaphragmatic hernia (168). Interestingly, TN-C expression is decreased after ventilating newborn mice (169), while TN-C is highly expressed in autopsied babies with BPD (170). It is likely that down-regulation of TN-C after (non-injurious) ventilation may negatively influence alveolar development and that the increase in TN-C expression fits with a lung injury repair response. Recent analysis of gene expression in repairing lungs of naphthalene-exposed mice showed a transient increase of TN-C expression in the lung whereas abortive repair in a transgenic model allowing ablation of all reparative cells resulted in a progressive accumulation of TN-C (171).

Infants with BPD have dysmorphic capillaries and a decreased expression of VEGF in the lungs (154). In the BALF of preterm infants developing BPD the level of VEGF was also decreased (172). The same findings were reported in premature baboons, mice and lambs developing BPD. Pulmonary expression of VEGF and one or more VEGF receptors were decreased (169, 173-175). In all these studies mechanical ventilation was combined with oxygen. Ventilation with air for 8 hours did not alter the expression of VEGF or its receptor in newborn mice (169). In contrast the expression of PDGF-A was decreased after ventilation with oxygen as well as after ventilation with room air (169). This may indicate that PDGF signaling is more related to stretch-induced lung injury than VEGF signaling.

SYNOPSIS

Mechanical ventilation is a major contributor to the complex and multifactorial pathogenesis of BPD. It initiates a pulmonary fetal inflammatory response. Often this lung inflammation is superimposed onto an intra-uterine inflammation and is enhanced by exposure to supplemental oxygen. Stretch alone or in combination with these initiators of lung injury dysregulate the precise temporal and spatial orchestration of molecular interactions, necessary for normal lung development and lung repair. This molecular dysregulation results in an arrest in alveolarisation and vascular development. The underlying mechanisms and pathways by which mechanical ventilation causes the abnormal lung development that characterize BPD, however, remains largely unknown.

REFERENCES

1. Doyle LW, Anderson PJ. Long-term outcomes of bronchopulmonary dysplasia. Semin Fetal Neonatal Med. 2009 Dec;14(6):391-5.
2. Northway WH, Jr., Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med. 1967 Feb 16; 276(7):357-68.
3. Pusey VA, Macpherson RI, Chernick V. Pulmonary fibroplasia following prolonged artificial ventilation of newborn infants. Can Med Assoc J. 1969 Mar 8;100(10):451-7.
4. Husain AN, Siddiqui NH, Stocker JT. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. Hum Pathol. 1998 Jul;29(7):710-7.
5. Coalson JJ. Pathology of new bronchopulmonary dysplasia. Semin Neonatol. 2003 Feb; 8(1):73-81.
6. Hillman NH, Kallapur SG, Pillow JJ, Moss TJ, Polglase GR, Nitsos I, et al. Airway injury from initiating ventilation in preterm sheep. Pediatr Res. Jan;67(1):60-5.
7. Thomson MA, Yoder BA, Winter VT, Martin H, Catland D, Siler-Khodr TM, et al. Treatment of immature baboons for 28 days with early nasal continuous positive airway pressure. Am J Respir Crit Care Med. 2004 May 1;169(9):1054-62.
8. Horbar JD, Badger GJ, Carpenter JH, Fanaroff AA, Kilpatrick S, LaCorte M, et al. Trends in mortality and morbidity for very low birth weight infants, 1991-1999. Pediatrics. 2002 Jul; 110(1 Pt 1):143-51.
9. Fanaroff AA, Hack M, Walsh MC. The NICHD neonatal research network: changes in practice and outcomes during the first 15 years. Semin Perinatol. 2003 Aug;27(4):281-7.
10. Srisuparp P, Marks JD, Khoshnood B, Schreiber MD. Predictive power of initial severity of pulmonary disease for subsequent development of bronchopulmonary dysplasia. Biol Neonate. 2003;84(1):31-6.
11. Smith VC, Zupancic JA, McCormick MC, Croen LA, Greene J, Escobar GJ, et al. Trends in severe bronchopulmonary dysplasia rates between 1994 and 2002. J Pediatr. 2005 Apr; 146(4):469-73.
12. Bhandari A, Bhandari V. Pathogenesis, pathology and pathophysiology of pulmonary sequelae of bronchopulmonary dysplasia in premature infants. Front Biosci. 2003 May 1; 8:e370-80.
13. Kinsella JP, Greenough A, Abman SH. Bronchopulmonary dysplasia. Lancet. 2006 Apr 29; 367(9520):1421-31.
14. Dreyfuss D, Soler P, Basset G, Saumon G. High inflation pressure pulmonary edema. Respective effects of high airway pressure, high tidal volume, and positive end-expiratory pressure. Am Rev Respir Dis. 1988 May;137(5):1159-64.
15. Hernandez LA, Peevy KJ, Moise AA, Parker JC. Chest wall restriction limits high airway pressure-induced lung injury in young rabbits. J Appl Physiol. 1989 May;66(5):2364-8.
16. McCallion N, Davis PG, Morley CJ. Volume-targeted versus pressure-limited ventilation in the neonate. Cochrane Database Syst Rev. 2005(3):CD003666.
17. Gorenflo M, Vogel M, Herbst L, Bassir C, Kattner E, Obladen M. Influence of clinical and ventilatory parameters on morphology of bronchopulmonary dysplasia. Pediatr Pulmonol. 1995 Apr;19(4):214-20.
18. Parker JC, Hernandez LA, Peevy KJ. Mechanisms of ventilator-induced lung injury. Crit Care Med. 1993 Jan;21(1):131-43.

19. Dreyfuss D, Saumon G. Ventilator-induced lung injury: lessons from experimental studies. Am J Respir Crit Care Med. 1998 Jan;157(1):294-323.
20. de Prost N, Roux D, Dreyfuss D, Ricard JD, Le Guludec D, Saumon G. Alveolar edema dispersion and alveolar protein permeability during high volume ventilation: effect of positive end-expiratory pressure. Intensive Care Med. 2007 Apr;33(4):711-7.
21. Peterson SW. Understanding the sequence of pulmonary injury in the extremely low birth weight, surfactant-deficient infant. Neonatal Netw. 2009 Jul-Aug;28(4):221-9; quiz 55-8.
22. Merrill JD, Ballard RA, Cnaan A, Hibbs AM, Godinez RI, Godinez MH, et al. Dysfunction of pulmonary surfactant in chronically ventilated premature infants. Pediatr Res. 2004 Dec;56(6):918-26.
23. Gajic O, Lee J, Doerr CH, Berrios JC, Myers JL, Hubmayr RD. Ventilator-induced cell wounding and repair in the intact lung. Am J Respir Crit Care Med. 2003 Apr 15;167(8): 1057-63.
24. Chu EK, Whitehead T, Slutsky AS. Effects of cyclic opening and closing at low- and high-volume ventilation on bronchoalveolar lavage cytokines. Crit Care Med. 2004 Jan;32(1): 168-74.
25. Muscedere JG, Mullen JB, Gan K, Slutsky AS. Tidal ventilation at low airway pressures can augment lung injury. Am J Respir Crit Care Med. 1994 May;149(5):1327-34.
26. Monkman SL, Andersen CC, Nahmias C, Ghaffer H, Bourgeois JM, Roberts RS, et al. Positive end-expiratory pressure above lower inflection point minimizes influx of activated neutrophils into lung. Crit Care Med. 2004 Dec;32(12):2471-5.
27. Bayle F, Guerin C, Debord S, Badet M, Lemasson S, Poupelin JC, et al. Assessment of airway closure from deflation lung volume-pressure curve: sigmoidal equation revisited. Intensive Care Med. 2006 Jun;32(6):894-8.
28. Rimensberger PC, Cox PN, Frndova H, Bryan AC. The open lung during small tidal volume ventilation: concepts of recruitment and "optimal" positive end-expiratory pressure. Crit Care Med. 1999 Sep;27(9):1946-52.
29. Slutsky AS. Ventilator-induced lung injury: from barotrauma to biotrauma. Respir Care. 2005 May;50(5):646-59.
30. Copland IB, Martinez F, Kavanagh BP, Engelberts D, McKerlie C, Belik J, et al. High tidal volume ventilation causes different inflammatory responses in newborn versus adult lung. Am J Respir Crit Care Med. 2004 Mar 15;169(6):739-48.
31. Uhlig S. Ventilation-induced lung injury and mechanotransduction: stretching it too far? Am J Physiol Lung Cell Mol Physiol. 2002 May;282(5):L892-6.
32. Yoder BA, Siler-Khodr T, Winter VT, Coalson JJ. High-frequency oscillatory ventilation: effects on lung function, mechanics, and airway cytokines in the immature baboon model for neonatal chronic lung disease. Am J Respir Crit Care Med. 2000 Nov;162(5):1867-76.
33. Tremblay LN, Miatto D, Hamid Q, Govindarajan A, Slutsky AS. Injurious ventilation induces widespread pulmonary epithelial expression of tumor necrosis factor-alpha and interleukin-6 messenger RNA. Crit Care Med. 2002 Aug;30(8):1693-700.
34. Pugin J, Dunn I, Jolliet P, Tassaux D, Magnenat JL, Nicod LP, et al. Activation of human macrophages by mechanical ventilation in vitro. Am J Physiol. 1998 Dec;275(6 Pt 1): L1040-50.
35. Ward PA, Lentsch AB. The acute inflammatory response and its regulation. Arch Surg. 1999 Jun;134(6):666-9.

36. Park WY, Goodman RB, Steinberg KP, Ruzinski JT, Radella F, 2nd, Park DR, et al. Cytokine balance in the lungs of patients with acute respiratory distress syndrome. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 1):1896-903.
37. Ward PA, Lentsch AB. Endogenous regulation of the acute inflammatory response. Mol Cell Biochem. 2002 May-Jun;234-235(1-2):225-8.
38. Puneet P, Moochhala S, Bhatia M. Chemokines in acute respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol. 2005 Jan;288(1):L3-15.
39. Jobe AH, Ikegami M. Mechanisms initiating lung injury in the preterm. Early Hum Dev. 1998 Nov;53(1):81-94.
40. Yi M, Jankov RP, Belcastro R, Humes D, Copland I, Shek S, et al. Opposing effects of 60% oxygen and neutrophil influx on alveologenesis in the neonatal rat. Am J Respir Crit Care Med. 2004 Dec 1;170(11):1188-96.
41. Lentsch AB, Shanley TP, Sarma V, Ward PA. In vivo suppression of NF-kappa B and preservation of I kappa B alpha by interleukin-10 and interleukin-13. J Clin Invest. 1997 Nov 15; 100(10):2443-8.
42. Ghezzi F, Gomez R, Romero R, Yoon BH, Edwin SS, David C, et al. Elevated interleukin-8 concentrations in amniotic fluid of mothers whose neonates subsequently develop bronchopulmonary dysplasia. Eur J Obstet Gynecol Reprod Biol. 1998 May;78(1):5-10.
43. Groneck P, Gotze-Speer B, Oppermann M, Eiffert H, Speer CP. Association of pulmonary inflammation and increased microvascular permeability during the development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluids of high-risk preterm neonates. Pediatrics. 1994 May;93(5):712-8.
44. Han B, Lodyga M, Liu M. Ventilator-induced lung injury: role of protein-protein interaction in mechanosensation. Proc Am Thorac Soc. 2005;2(3):181-7.
45. Liu M, Post M. Invited review: mechanochemical signal transduction in the fetal lung. J Appl Physiol. 2000 Nov;89(5):2078-84.
46. Liu M, Tanswell AK, Post M. Mechanical force-induced signal transduction in lung cells. Am J Physiol. 1999 Oct;277(4 Pt 1):L667-83.
47. Zamir E, Geiger B. Molecular complexity and dynamics of cell-matrix adhesions. J Cell Sci. 2001 Oct;114(Pt 20):3583-90.
48. Shafrir Y, Forgacs G. Mechanotransduction through the cytoskeleton. Am J Physiol Cell Physiol. 2002 Mar;282(3):C479-86.
49. Li YS, Haga JH, Chien S. Molecular basis of the effects of shear stress on vascular endothelial cells. J Biomech. 2005 Oct;38(10):1949-71.
50. Chen KD, Li YS, Kim M, Li S, Yuan S, Chien S, et al. Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc. J Biol Chem. 1999 Jun 25;274(26):18393-400.
51. Correa-Meyer E, Pesce L, Guerrero C, Sznajder JI. Cyclic stretch activates ERK1/2 via G proteins and EGFR in alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2002 May;282(5):L883-91.
52. Uhlig U, Haitsma JJ, Goldmann T, Poelma DL, Lachmann B, Uhlig S. Ventilation-induced activation of the mitogen-activated protein kinase pathway. Eur Respir J. 2002 Oct;20(4): 946-56.
53. Copland IB, Post M. Stretch-activated signaling pathways responsible for early response gene expression in fetal lung epithelial cells. J Cell Physiol. 2007 Jan;210(1):133-43.

54. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature. 2001 Mar 1; 410(6824):37-40.
55. Syeda F, Liu HY, Tullis E, Liu M, Slutsky AS, Zhang H. Differential signaling mechanisms of HNP-induced IL-8 production in human lung epithelial cells and monocytes. J Cell Physiol. 2008 Mar;214(3):820-7.
56. Vlahakis NE, Schroeder MA, Limper AH, Hubmayr RD. Stretch induces cytokine release by alveolar epithelial cells in vitro. Am J Physiol. 1999 Jul;277(1 Pt 1):L167-73.
57. Oudin S, Pugin J. Role of MAP kinase activation in interleukin-8 production by human BEAS-2B bronchial epithelial cells submitted to cyclic stretch. Am J Respir Cell Mol Biol. 2002 Jul;27(1):107-14.
58. Ji B, Chen XQ, Misek DE, Kuick R, Hanash S, Ernst S, et al. Pancreatic gene expression during the initiation of acute pancreatitis: identification of EGR-1 as a key regulator. Physiol Genomics. 2003 Jun 24;14(1):59-72.
59. Held HD, Boettcher S, Hamann L, Uhlig S. Ventilation-induced chemokine and cytokine release is associated with activation of nuclear factor-kappaB and is blocked by steroids. Am J Respir Crit Care Med. 2001 Mar;163(3 Pt 1):711-6.
60. Collins I, Garrett MD. Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors. Curr Opin Pharmacol. 2005 Aug;5(4):366-73.
61. Schafer KA. The cell cycle: a review. Vet Pathol. 1998 Nov;35(6):461-78.
62. Park MT, Lee SJ. Cell cycle and cancer. J Biochem Mol Biol. 2003 Jan 31;36(1):60-5.
63. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. 2003 Jun;36(3):131-49.
64. Coqueret O. New targets for viral cyclins. Cell Cycle. 2003 Jul-Aug;2(4):293-5.
65. Johnson DG, Walker CL. Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol. 1999;39:295-312.
66. Harbour JW, Luo RX, Dei Santi A, Postigo AA, Dean DC. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell. 1999 Sep 17;98(6):859-69.
67. Lundberg AS, Weinberg RA. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol Cell Biol. 1998 Feb;18(2):753-61.
68. Knudsen ES, Knudsen KE. Retinoblastoma tumor suppressor: where cancer meets the cell cycle. Exp Biol Med (Maywood). 2006 Jul;231(7):1271-81.
69. Parry D, Bates S, Mann DJ, Peters G. Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product. Embo J. 1995 Feb 1;14(3):503-11.
70. McConnell BB, Gregory FJ, Stott FJ, Hara E, Peters G. Induced expression of p16(INK4a) inhibits both CDK4- and CDK2-associated kinase activity by reassortment of cyclin-CDK-inhibitor complexes. Mol Cell Biol. 1999 Mar;19(3):1981-9.
71. Soos TJ, Kiyokawa H, Yan JS, Rubin MS, Giordano A, DeBlasio A, et al. Formation of p27-CDK complexes during the human mitotic cell cycle. Cell Growth Differ. 1996 Feb;7(2): 135-46.
72. Chen J, Jackson PK, Kirschner MW, Dutta A. Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. Nature. 1995 Mar 23;374(6520):386-8.

73. Guan KL, Jenkins CW, Li Y, Nichols MA, Wu X, O'Keefe CL, et al. Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. Genes Dev. 1994 Dec 15;8(24):2939-52.
74. Hirai H, Roussel MF, Kato JY, Ashmun RA, Sherr CJ. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol Cell Biol. 1995 May;15(5):2672-81.
75. Nakanishi M, Robetorye RS, Adami GR, Pereira-Smith OM, Smith JR. Identification of the active region of the DNA synthesis inhibitory gene p21Sdi1/CIP1/WAF1. Embo J. 1995 Feb 1;14(3):555-63.
76. Luo Y, Hurwitz J, Massague J. Cell-cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. Nature. 1995 May 11;375(6527):159-61.
77. Kitterman JA. The effects of mechanical forces on fetal lung growth. Clin Perinatol. 1996 Dec;23(4):727-40.
78. Hooper SB, Harding R. Fetal lung liquid: a major determinant of the growth and functional development of the fetal lung. Clin Exp Pharmacol Physiol. 1995 Apr;22(4):235-47.
79. Scarpelli EM, Condorelli S, Cosmi EV. Lamb fetal pulmonary fluid. I. Validation and significance of method for determination of volume and volume change. Pediatr Res. 1975 Apr; 9(4):190-5.
80. Miller AA, Hooper SB, Harding R. Role of fetal breathing movements in control of fetal lung distension. J Appl Physiol. 1993 Dec;75(6):2711-7.
81. Harding R. Fetal breathing movements. In *the Lung: Scientific foundations (2nd ed.),* edited by Crystal RG, West JB, Banes PJ, and Weiber ER. Philedelphia, PA: Lippincott-Raven, 1997, p.2093-2104. Crystal RG WJ, Banes PJ, and Weiber ER, editor.
82. Liggins GC, Vilos GA, Campos GA, Kitterman JA, Lee CH. The effect of bilateral thoracoplasty on lung development in fetal sheep. J Dev Physiol. 1981 Aug;3(4):275-82.
83. Nagai A, Thurlbeck WM, Jansen AH, Ioffe S, Chernick V. The effect of chronic biphrenectomy on lung growth and maturation in fetal lambs. Morphologic and morphometric studies. Am Rev Respir Dis. 1988 Jan;137(1):167-72.
84. Alcorn D, Adamson TM, Lambert TF, Maloney JE, Ritchie BC, Robinson PM. Morphological effects of chronic tracheal ligation and drainage in the fetal lamb lung. J Anat. 1977 Jul; 123(Pt 3):649-60.
85. Nardo L, Maritz G, Harding R, Hooper SB. Changes in lung structure and cellular division induced by tracheal obstruction in fetal sheep. Exp Lung Res. 2000 Mar;26(2):105-19.
86. Joe P, Wallen LD, Chapin CJ, Lee CH, Allen L, Han VK, et al. Effects of mechanical factors on growth and maturation of the lung in fetal sheep. Am J Physiol. 1997 Jan;272(1 Pt 1): L95-105.
87. De Paepe ME, Johnson BD, Papadakis K, Luks FI. Lung growth response after tracheal occlusion in fetal rabbits is gestational age-dependent. Am J Respir Cell Mol Biol. 1999 Jul; 21(1):65-76.
88. Uhal BD. Cell cycle kinetics in the alveolar epithelium. Am J Physiol. 1997 Jun;272(6 Pt 1):L1031-45.
89. Flecknoe S, Harding R, Maritz G, Hooper SB. Increased lung expansion alters the proportions of type I and type II alveolar epithelial cells in fetal sheep. Am J Physiol Lung Cell Mol Physiol. 2000 Jun;278(6):L1180-5.
90. Xu J, Liu M, Tanswell AK, Post M. Mesenchymal determination of mechanical strain-induced fetal lung cell proliferation. Am J Physiol. 1998 Sep;275(3 Pt 1):L545-50.

91. Sanchez-Esteban J, Wang Y, Cicchiello LA, Rubin LP. Cyclic mechanical stretch inhibits cell proliferation and induces apoptosis in fetal rat lung fibroblasts. Am J Physiol Lung Cell Mol Physiol. 2002 Mar;282(3):L448-56.
92. McGrath SA. Induction of p21WAF/CIP1 during hyperoxia. Am J Respir Cell Mol Biol. 1998 Feb;18(2):179-87.
93. Corroyer S, Maitre B, Cazals V, Clement A. Altered regulation of G1 cyclins in oxidant-induced growth arrest of lung alveolar epithelial cells. Accumulation of inactive cyclin E-DCK2 complexes. J Biol Chem. 1996 Oct 11;271(41):25117-25.
94. Bonikos DS, Bensch KG, Northway WH, Jr., Edwards DK. Bronchopulmonary dysplasia: the pulmonary pathologic sequel of necrotizing bronchiolitis and pulmonary fibrosis. Hum Pathol. 1976 Nov;7(6):643-66.
95. O'Reilly MA, Watkins RH, Staversky RJ, Maniscalco WM. Induced p21Cip1 in premature baboons with CLD: implications for alveolar hypoplasia. Am J Physiol Lung Cell Mol Physiol. 2003 Oct;285(4):L964-71.
96. Das KC, Ravi D. Altered expression of cyclins and cdks in premature infant baboon model of bronchopulmonary dysplasia. Antioxid Redox Signal. 2004 Feb;6(1):117-27.
97. Buckley S, Bui KC, Hussain M, Warburton D. Dynamics of TGF-beta 3 peptide activity during rat alveolar epithelial cell proliferative recovery from acute hyperoxia. Am J Physiol. 1996 Jul;271(1 Pt 1):L54-60.
98. Reynisdottir I, Polyak K, Iavarone A, Massague J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes Dev. 1995 Aug 1;9(15):1831-45.
99. Yasuhara S, Asai A, Sahani ND, Martyn JA. Mitochondria, endoplasmic reticulum, and alternative pathways of cell death in critical illness. Crit Care Med. 2007 Sep;35(9 Suppl):S488-95.
100. Scavo LM, Ertsey R, Chapin CJ, Allen L, Kitterman JA. Apoptosis in the development of rat and human fetal lungs. Am J Respir Cell Mol Biol. 1998 Jan;18(1):21-31.
101. Chipuk JE, Green DR. Do inducers of apoptosis trigger caspase-independent cell death? Nat Rev Mol Cell Biol. 2005 Mar;6(3):268-75.
102. Lionetti V, Recchia FA, Ranieri VM. Overview of ventilator-induced lung injury mechanisms. Curr Opin Crit Care. 2005 Feb;11(1):82-6.
103. Afford S, Randhawa S. Apoptosis. Mol Pathol. 2000 Apr;53(2):55-63.
104. Nicotera P, Leist M, Ferrando-May E. Apoptosis and necrosis: different execution of the same death. Biochem Soc Symp. 1999;66:69-73.
105. Kroemer G, Martin SJ. Caspase-independent cell death. Nat Med. 2005 Jul;11(7):725-30.
106. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol. 1999;15:269-90.
107. Drakopanagiotakis F, Xifteri A, Polychronopoulos V, Bouros D. Apoptosis in lung injury and fibrosis. Eur Respir J. 2008 Dec;32(6):1631-8.
108. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004 Jan 23;116(2):205-19.
109. Jaattela M. Multiple cell death pathways as regulators of tumour initiation and progression. Oncogene. 2004 Apr 12;23(16):2746-56.
110. Leist M, Jaattela M. Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol. 2001 Aug;2(8):589-98.

111. Mantell LL, Lee PJ. Signal transduction pathways in hyperoxia-induced lung cell death. Mol Genet Metab. 2000 Sep-Oct;71(1-2):359-70.
112. Liu X, Van Vleet T, Schnellmann RG. The role of calpain in oncotic cell death. Annu Rev Pharmacol Toxicol. 2004;44:349-70.
113. Martin TR, Hagimoto N, Nakamura M, Matute-Bello G. Apoptosis and epithelial injury in the lungs. Proc Am Thorac Soc. 2005;2(3):214-20.
114. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet. 2009;43:67-93.
115. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. N Engl J Med. 2009 Oct 15; 361(16):1570-83.
116. Huang J, Klionsky DJ. Autophagy and human disease. Cell Cycle. 2007 Aug 1;6(15): 1837-49.
117. Schittny JC, Djonov V, Fine A, Burri PH. Programmed cell death contributes to postnatal lung development. Am J Respir Cell Mol Biol. 1998 Jun;18(6):786-93.
118. Kresch MJ, Christian C, Wu F, Hussain N. Ontogeny of apoptosis during lung development. Pediatr Res. 1998 Mar;43(3):426-31.
119. Bruce MC, Honaker CE, Cross RJ. Lung fibroblasts undergo apoptosis following alveolarization. Am J Respir Cell Mol Biol. 1999 Feb;20(2):228-36.
120. Bardales RH, Xie SS, Schaefer RF, Hsu SM. Apoptosis is a major pathway responsible for the resolution of type II pneumocytes in acute lung injury. Am J Pathol. 1996 Sep;149(3): 845-52.
121. Lee HS, Wang Y, Maciejewski BS, Esho K, Fulton C, Sharma S, et al. Interleukin-10 protects cultured fetal rat type II epithelial cells from injury induced by mechanical stretch. Am J Physiol Lung Cell Mol Physiol. 2008 Feb;294(2):L225-32.
122. Mokres LM, Parai K, Hilgendorff A, Ertsey R, Alvira CM, Rabinovitch M, et al. 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. Jan; 298(1):L23-35.
123. Allison BJ, Crossley KJ, Flecknoe SJ, Davis PG, Morley CJ, Hooper SB. Ventilation and oxygen: cumulative effects of oxygen on ventilation induced lung injury. Pediatr Res. 2009 Dec 11.
124. Del Riccio V, van Tuyl M, Post M. Apoptosis in lung development and neonatal lung injury. Pediatr Res. 2004 Feb;55(2):183-9.
125. De Paepe ME, Mao Q, Luks FI. Expression of apoptosis-related genes after fetal tracheal occlusion in rabbits. J Pediatr Surg. 2004 Nov;39(11):1616-25.
126. De Paepe ME, Mao Q, Embree-Ku M, Rubin LP, Luks FI. Fas/FasL-mediated apoptosis in perinatal murine lungs. Am J Physiol Lung Cell Mol Physiol. 2004 Oct;287(4):L730-42.
127. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. Am J Respir Crit Care Med. 1999 Oct;160(4):1333-46.
128. Speer CP. Inflammation and bronchopulmonary dysplasia: a continuing story. Semin Fetal Neonatal Med. 2006 Oct;11(5):354-62.
129. Lecart C, Cayabyab R, Buckley S, Morrison J, Kwong KY, Warburton D, et al. Bioactive transforming growth factor-beta in the lungs of extremely low birthweight neonates predicts the need for home oxygen supplementation. Biol Neonate. 2000 May;77(4):217-23.

130. Alejandre-Alcazar MA, Kwapiszewska G, Reiss I, Amarie OV, Marsh LM, Sevilla-Perez J, et al. Hyperoxia modulates TGF-beta/BMP signaling in a mouse model of bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol. 2007 Feb;292(2):L537-49.
131. Moustakas A, Heldin CH. The regulation of TGFbeta signal transduction. Development. 2009 Nov;136(22):3699-714.
132. Hagimoto N, Kuwano K, Inoshima I, Yoshimi M, Nakamura N, Fujita M, et al. TGF-beta 1 as an enhancer of Fas-mediated apoptosis of lung epithelial cells. J Immunol. 2002 Jun 15; 168(12):6470-8.
133. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther. 2002 Jun;1(8):639-49.
134. Pelosi P, Rocco PR. Effects of mechanical ventilation on the extracellular matrix. Intensive Care Med. 2008 Apr;34(4):631-9.
135. Mariani TJ, Sandefur S, Pierce RA. Elastin in lung development. Exp Lung Res. 1997 Mar-Apr;23(2):131-45.
136. Pierce RA, Mariencheck WI, Sandefur S, Crouch EC, Parks WC. Glucocorticoids upregulate tropoelastin expression during late stages of fetal lung development. Am J Physiol. 1995 Mar;268(3 Pt 1):L491-500.
137. Mariani TJ, Crouch E, Roby JD, Starcher B, Pierce RA. Increased elastin production in experimental granulomatous lung disease. Am J Pathol. 1995 Oct;147(4):988-1000.
138. Wendel DP, Taylor DG, Albertine KH, Keating MT, Li DY. Impaired distal airway development in mice lacking elastin. Am J Respir Cell Mol Biol. 2000 Sep;23(3):320-6.
139. Nakamura T, Lozano PR, Ikeda Y, Iwanaga Y, Hinek A, Minamisawa S, et al. Fibulin-5/DANCE is essential for elastogenesis in vivo. Nature. 2002 Jan 10;415(6868):171-5.
140. Hirai M, Ohbayashi T, Horiguchi M, Okawa K, Hagiwara A, Chien KR, et al. Fibulin-5/DANCE has an elastogenic organizer activity that is abrogated by proteolytic cleavage in vivo. J Cell Biol. 2007 Mar 26;176(7):1061-71.
141. Maki JM, Sormunen R, Lippo S, Kaarteenaho-Wiik R, Soininen R, Myllyharju J. Lysyl oxidase is essential for normal development and function of the respiratory system and for the integrity of elastic and collagen fibers in various tissues. Am J Pathol. 2005 Oct;167(4): 927-36.
142. Kaarteenaho-Wiik R, Kinnula V, Herva R, Paakko P, Pollanen R, Soini Y. Distribution and mRNA expression of tenascin-C in developing human lung. Am J Respir Cell Mol Biol. 2001 Sep;25(3):341-6.
143. Roth-Kleiner M, Hirsch E, Schittny JC. Fetal lungs of tenascin-C-deficient mice grow well, but branch poorly in organ culture. Am J Respir Cell Mol Biol. 2004 Mar;30(3):360-6.
144. Young SL, Chang LY, Erickson HP. Tenascin-C in rat lung: distribution, ontogeny and role in branching morphogenesis. Dev Biol. 1994 Feb;161(2):615-25.
145. Chiquet-Ehrismann R. Tenascins. Int J Biochem Cell Biol. 2004 Jun;36(6):986-90.
146. Zhao Y, Young SL. TGF-beta regulates expression of tenascin alternative-splicing isoforms in fetal rat lung. Am J Physiol. 1995 Feb;268(2 Pt 1):L173-80.
147. Hislop AA. Airway and blood vessel interaction during lung development. J Anat. 2002 Oct;201(4):325-34.
148. Le Cras TD, Kim DH, Gebb S, Markham NE, Shannon JM, Tuder RM, et al. Abnormal lung growth and the development of pulmonary hypertension in the Fawn-Hooded rat. Am J Physiol. 1999 Oct;277(4 Pt 1):L709-18.

149. le Cras TD, Markham NE, Morris KG, Ahrens CR, McMurtry IF, Abman SH. Neonatal dexamethasone treatment increases the risk for pulmonary hypertension in adult rats. Am J Physiol Lung Cell Mol Physiol. 2000 Apr;278(4):L822-9.
150. Randell SH, Mercer RR, Young SL. Neonatal hyperoxia alters the pulmonary alveolar and capillary structure of 40-day-old rats. Am J Pathol. 1990 Jun;136(6):1259-66.
151. Parker TA, Abman SH. The pulmonary circulation in bronchopulmonary dysplasia. Semin Neonatol. 2003 Feb;8(1):51-61.
152. Margraf LR, Tomashefski JF, Jr., Bruce MC, Dahms BB. Morphometric analysis of the lung in bronchopulmonary dysplasia. Am Rev Respir Dis. 1991 Feb;143(2):391-400.
153. Thibeault DW, Haney B. Lung volume, pulmonary vasculature, and factors affecting survival in congenital diaphragmatic hernia. Pediatrics. 1998 Feb;101(2):289-95.
154. Bhatt AJ, Pryhuber GS, Huyck H, Watkins RH, Metlay LA, Maniscalco WM. Disrupted pulmonary vasculature and decreased vascular endothelial growth factor, Flt-1, and TIE-2 in human infants dying with bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 1):1971-80.
155. Jakkula M, Le Cras TD, Gebb S, Hirth KP, Tuder RM, Voelkel NF, et al. Inhibition of angiogenesis decreases alveolarization in the developing rat lung. Am J Physiol Lung Cell Mol Physiol. 2000 Sep;279(3):L600-7.
156. McGrath-Morrow SA, Cho C, Cho C, Zhen L, Hicklin DJ, Tuder RM. Vascular endothelial growth factor receptor 2 blockade disrupts postnatal lung development. Am J Respir Cell Mol Biol. 2005 May;32(5):420-7.
157. Thebaud B, Ladha F, Michelakis ED, Sawicka M, Thurston G, Eaton F, et al. Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. Circulation. 2005 Oct 18;112(16):2477-86.
158. Kunig AM, Balasubramaniam V, Markham NE, Morgan D, Montgomery G, Grover TR, et al. Recombinant human VEGF treatment enhances alveolarization after hyperoxic lung injury in neonatal rats. Am J Physiol Lung Cell Mol Physiol. 2005 Oct;289(4):L529-35.
159. Bostrom H, Willetts K, Pekny M, Leveen P, Lindahl P, Hedstrand H, et al. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. Cell. 1996 Jun 14;85(6):863-73.
160. Betsholtz C, Karlsson L, Lindahl P. Developmental roles of platelet-derived growth factors. Bioessays. 2001 Jun;23(6):494-507.
161. Lasky JA, Tonthat B, Liu JY, Friedman M, Brody AR. Upregulation of the PDGF-alpha receptor precedes asbestos-induced lung fibrosis in rats. Am J Respir Crit Care Med. 1998 May; 157(5 Pt 1):1652-7.
162. Nakamura T, Liu M, Mourgeon E, Slutsky A, Post M. Mechanical strain and dexamethasone selectively increase surfactant protein C and tropoelastin gene expression. Am J Physiol Lung Cell Mol Physiol. 2000 May;278(5):L974-80.
163. Joyce BJ, Wallace MJ, Pierce RA, Harding R, Hooper SB. Sustained changes in lung expansion alter tropoelastin mRNA levels and elastin content in fetal sheep lungs. Am J Physiol Lung Cell Mol Physiol. 2003 Apr;284(4):L643-9.
164. Chen CM, Wang LF, Chou HC, Lang YD. Oligohydramnios decreases platelet-derived growth factor expression in fetal rat lungs. Neonatology. 2007;92(3):187-93.

165. Haidar A, Ryder TA, Wigglesworth JS. Failure of elastin development in hypoplastic lungs associated with oligohydramnios: an electronmicroscopic study. Histopathology. 1991 May;18(5):471-3.
166. Bland RD, Ertsey R, Mokres LM, Xu L, Jacobson BE, Jiang S, et al. Mechanical ventilation uncouples synthesis and assembly of elastin and increases apoptosis in lungs of newborn mice. Prelude to defective alveolar septation during lung development? Am J Physiol Lung Cell Mol Physiol. 2008 Jan;294(1):L3-14.
167. Hasaneen NA, Zucker S, Cao J, Chiarelli C, Panettieri RA, Foda HD. Cyclic mechanical strain-induced proliferation and migration of human airway smooth muscle cells: role of EMMPRIN and MMPs. Faseb J. 2005 Sep;19(11):1507-9.
168. Chinoy MR, Miller SA. Relevance of tenascin-C and matrix metalloproteinases in vascular abnormalities in murine hypoplastic lungs. Biol Neonate. 2006;90(3):185-96.
169. Bland RD, Mokres LM, Ertsey R, Jacobson BE, Jiang S, Rabinovitch M, et al. Mechanical ventilation with 40% oxygen reduces pulmonary expression of genes that regulate lung development and impairs alveolar septation in newborn mice. Am J Physiol Lung Cell Mol Physiol. 2007 Nov;293(5):L1099-110.
170. Kaarteenaho-Wiik R, Kinnula VL, Herva R, Soini Y, Pollanen R, Paakko P. Tenascin-C is highly expressed in respiratory distress syndrome and bronchopulmonary dysplasia. J Histochem Cytochem. 2002 Mar;50(3):423-31.
171. Snyder JC, Zemke AC, Stripp BR. Reparative capacity of airway epithelium impacts deposition and remodeling of extracellular matrix. Am J Respir Cell Mol Biol. 2009 Jun;40(6): 633-42.
172. Been JV, Debeer A, van Iwaarden JF, Kloosterboer N, Passos VL, Naulaers G, et al. Early alterations of growth factor patterns in bronchoalveolar lavage fluid from preterm infants developing bronchopulmonary dysplasia. Pediatr Res. Jan;67(1):83-9.
173. Tambunting F, Beharry KD, Waltzman J, Modanlou HD. Impaired lung vascular endothelial growth factor in extremely premature baboons developing bronchopulmonary dysplasia/ chronic lung disease. J Investig Med. 2005 Jul;53(5):253-62.
174. Maniscalco WM, Watkins RH, Pryhuber GS, Bhatt A, Shea C, Huyck H. Angiogenic factors and alveolar vasculature: development and alterations by injury in very premature baboons. Am J Physiol Lung Cell Mol Physiol. 2002 Apr;282(4):L811-23.
175. Bland RD, Xu L, Ertsey R, Rabinovitch M, Albertine KH, Wynn KA, et al. Dysregulation of pulmonary elastin synthesis and assembly in preterm lambs with chronic lung disease. Am J Physiol Lung Cell Mol Physiol. 2007 Jun;292(6):L1370-84.

Chapter 2

Scope and outline of this thesis

The clinical relevance of Bronchopulmonary dysplasia

BPD is most commonly defined as an ongoing requirement for supplemental oxygen and/or respiratory support at 36 weeks post-menstrual age (1). It is a chronic respiratory disease that develops as a consequence of neonatal lung injury. The inciting factor of BPD is often pre- maturity with respiratory distress syndrome (RDS) from surfactant deficiency. Mechanical ventilation is one of the most important risk factors. Patients with BPD remain oxygen dependent for many months and in the first two years they frequently require hospital readmission and use more medication than patients without BPD (2, 3). The most severely affected children may remain symptomatic and have evidence of airway obstruction as adults (4). Besides respiratory morbidity, BPD is frequently associated with other conditions related to preterm birth, including growth retardation, feeding difficulties, pulmonary hypertension, neurodevelopmental delay, hearing defects, and retinopathy of prematurity. Financial and emotional costs of BPD are tremendous (5, 6). Despite ongoing researches for the optimal ventilation strategy and drug therapies to prevent BPD or to mitigate its course, BPD is still a major complication of premature birth.

Why basic research?

As mentioned above, it may be evident that greater insight into molecular pathways is needed to develop new strategies or therapies. The underlying mechanisms by which riskfactors such as mechanical ventilation, inflammation and oxygen, contribute to the pathogenesis of BPD are difficult to study in the clinical setting, where the role of each risk factor cannot be easily isolated from each other. Moreover, lung material of preterm infants to study molecular pathways involved in the pathogenesis of BPD is very limited. In this thesis we used a newborn rat model. This experimental approach takes advantage of the fact that rat lungs at birth have a saccular appearance, similar to the preterm neonate, and alveolarization in rats occurs postnatally between day 4 and day 21 (7). The newborn rat model enables us to examine the impact of each risk factor alone or in combination on genes and proteins that regulate lung growth and development.

This thesis

Lungs of preterm infants are in the early phases of lung development (canalicular and saccular phases of lung development). The alveolar and capillary development is inhibited following exposure to the injurious effects of mechanical ventilation, oxygen exposure, and inflammation. Insight in molecular pathways leading to arrest of alveologenesis and vasculogenesis, the hallmark of BPD, may have therapeutic potential for the prevention -or treatment- of BPD.

The major aims of this thesis are:

- To investigate the effect of different tidal volumes on the cytokine/chemokine production in newborn rat lungs.
- To investigate if mechanical ventilation with 50% oxygen superimposed on a systemic infection will enhance the cytokine/chemokine production in comparison with ventilation with room air without a systemic infection.
- To study which apoptotic pathways are induced *in vivo* by (prolonged) mechanical ventilation and *in vitro* by cell stretch.
- To determine the effect of (prolonged) mechanical ventilation on lung cell cycle regulators, proliferation and alveolarization *in vitro* and *in vivo*.
- To investigate how (prolonged) mechanical ventilation influences the expression of angiogenic and matrix molecules *in vivo*.

Stretching and alveolar collapse and re-expansion of alveoli is related to lung injury. **In chapter 3** we assessed the effect of low, moderate and high tidal volume (V_T) ventilation on cytokine/chemokine production. We hypothesized that continuous cyclic (over)stretching of the primitive airsacs would adversely affect cytokine/chemokine production and the adverse effect would be stretch-amplitude dependent. Secondly, we studied the effect of ventilation with controlled oxygen superimposed on a systemic inflammation on this cytokine/chemokine production.

Increased pro-inflammatory cytokines/chemokines production, induced by mechanical ventilation, hyperoxia and inflammation, is associated with activation of apoptotic pathways. Apoptosis is a highly orchestrated form of cell death. Lung cells undergo apoptosis as part of normal lung development and repair after lung injury. Several *in vivo* and *in vitro* studies suggest contribution of apoptosis to the pathophysiology of BPD due to an imbalance between anti-apoptotic and pro-apoptotic signaling pathways. **Chapter 4** is a review about the timing and apoptotic events during lung development and the pathogenesis of BPD with particular focus upon apoptotic pathways activated by mechanical ventilation, hyperoxia and inflammation. **Chapter 5** describes more in detail how apoptotic pathways are activated in newborn rats by prolonged mechanical ventilation and in isolated epithelial and fibroblast cells, subjected to continuous cyclic stretch. **Chapter 6** continues with the topic on lung cell proliferation. Besides apoptosis, tissue remodeling during lung development and after injury also requires well-coordinated regulation of cell proliferation. There is ample evidence that dysregulated cell proliferation, resulting from disruption of cell cycle control, promotes tumor progression in many cancers. If dysregulated cell proliferation is also involved in the pathogenesis of BPD is mostly unknown.

Also interactions between epithelial and mesenchymal cells play an important role both in lung development and in fibrotic disorders, including BPD. It is evident that

cytokines control these processes. Cytokines control the expression of several extracellular matrix (ECM) matrix molecules. In **Chapter 7** we investigate the influence of (prolonged) mechanical ventilation on the expression of angiogenic molecules (VEGF, PDGF, PECAM) and it receptors that regulate alveolarization and matrix molecules, principally the ones that regulate elastin synthesis and assembly (tropoelastin, fibulin5, lysyl oxydase). Finally the results of our findings are discussed and summarized in **Chapter 8 and 9**.

REFERENCES

1. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001 Jun; 163(7):1723-9.
2. Hennessy EM, Bracewell MA, Wood N, Wolke D, Costeloe K, Gibson A, et al. Respiratory health in pre-school and school age children following extremely preterm birth. Arch Dis Child. 2008 Dec;93(12):1037-43.
3. Baraldi E, Carraro S, Filippone M. Bronchopulmonary dysplasia: definitions and long-term respiratory outcome. Early Hum Dev. 2009 Oct;85(10 Suppl):S1-3.
4. Northway WH, Jr., Moss RB, Carlisle KB, Parker BR, Popp RL, Pitlick PT, et al. Late pulmonary sequelae of bronchopulmonary dysplasia. N Engl J Med. 1990 Dec 27;323(26): 1793-9.
5. McAleese KA, Knapp MA, Rhodes TT. Financial and emotional cost of bronchopulmonary dysplasia. Clin Pediatr (Phila). 1993 Jul;32(7):393-400.
6. Ireys HT, Anderson GF, Shaffer TJ, Neff JM. Expenditures for care of children with chronic illnesses enrolled in the Washington State Medicaid program, fiscal year 1993. Pediatrics. 1997 Aug;100(2 Pt 1):197-204.
7. Roth-Kleiner M, Ridsdale R, Cao L, Kuliszewski M, Tseu I, McKerlie C, et al. Lipopolysaccharide exposure modifies high tidal volume ventilation-induced proinflammatory mediator expression in newborn rat lungs. Pediatr Res. 2007 Feb;61(2):191-6.

Chapter 3

Inflammatory Response to Oxygen and Endotoxin in Newborn rat Lung ventilated with Low Tidal Volume

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ABSTRACT

Herein we determined the contribution of mechanical ventilation, hyperoxia and inflammation, individually or combined, to the cytokine/chemokine response of the neonatal lung. Eight day old rats were ventilated for 8 hours with low (~3.5 ml.kg-1), moderate (~12.5 ml.kg-1) or high (~25 ml.kg-1) tidal volumes (V_T) and the cytokine/chemokine response was measured. Next, we tested whether low V_T ventilation with 50% oxygen or a pre-existing inflammation induced by lipopolysaccharide (LPS) would modify this response. High, moderate and low V_T ventilation significantly elevated CXCL-2 and IL-6 mRNA levels. Low V_T ventilation with 50% oxygen significantly increased IL-6 and CXCL-2 expression vs. low V_T ventilation alone. LPS pre-treatment combined with low V_T ventilation with 50% oxygen amplified IL-6 mRNA expression when compared to low V_T alone or low V_T+50% O_2 treatment. In contrast, low V_T-upregulated CXCL-2 levels were reduced to non-ventilated levels when LPS-treated newborn rats were ventilated with 50% oxygen. Thus, low V_T ventilation triggers the expression of acute phase cytokines and CXC chemokines in newborn rat lung, which is amplified by oxygen but not by a pre-existing inflammation. Depending on the individual cytokine or chemokine, the combination of both oxygen and inflammation intensifies or abrogates the low V_T-induced inflammatory response.

INTRODUCTION

Bronchopulmonary dysplasia (BPD) remains the most important cause of respiratory morbidity in very low birth weight infants. Mechanical ventilation (MV), intra-uterine infections and oxidative stress upregulate pro-inflammatory cytokines/chemokines including interleukin(IL)-1β, IL-6 and IL-8 (1). Elevated concentrations of these cytokines/chemokines in amniotic fluid and bronchoalveolar lavage fluid (BALF) have been associated with BPD (2, 3). The contribution of each risk factor, alone or combined, to the inflammatory response remains to be determined.

Ample animal studies have suggested that high frequency oscillatory ventilation (HFOV) is less injurious compared to conventional ventilation (4, 5). However, in the baboon model of BPD impaired alveolarization and capillary development occurred in spite of appropriate oxygenation and use of HFOV (4). MV with moderate and high tidal volumes increased lung cytokine/chemokine response to systemic endotoxin in rabbits (6) and newborn rats (7). Oxidant injury alone can produce the pathologic features of BPD (8). Inflammatory cells such as monocytes and neutrophils are primary contributors to the oxygen-induced lung injury (9, 10). Other animal studies have investigated the contributions of oxygen exposure and MV alone or in combination. In term ventilated piglets hyperoxia caused less lung damage than hyperoxia combined with hyperventilation, but more than hyperventilation alone (11). Premature baboons ventilated with the minimum necessary supplemental oxygen had significant less damage than those ventilated with 100% oxygen (12), but alveolarization and capillary development was still impaired (4).

To our knowledge, no previous study has evaluated the combination of MV, hyperoxia and inflammation. Therefore, we first assessed the effect of low, moderate and high tidal volume (V_T) ventilation on cytokine/chemokine production. To mimic the clinical situation we used a newborn rat model (7). Rat lungs at birth have a saccular appearance, similar to the preterm neonate, and alveolarization in rats occurs postnatally between P4 and P21. High V_T ventilation has been reported to cause injury in newborn rat lung (13, 14) and was included as positive control. We hypothesized that continuous cyclic (over)stretching of the primitive airsacs would adversely affect cytokine/chemokine production and the adverse effect would be stretch-amplitude dependent. Secondly, we assessed the effect of low V_T ventilation with controlled oxygen superimposed on a systemic inflammation on cytokine/chemokine production. To induce a mild systemic inflammation we pre-treated the newborn rats with lipopolysaccharide (LPS) (7). We hypothesized that low V_T ventilation with 50% oxygen superimposed on a relatively mild systemic inflammation would enhance the adverse inflammatory mediator production by low tidal volume alone.

METHODS

Animals: In two series of experiments newborn (postnatal day 8) Spraque-Dawley rats (average weight 16.7 ± 1.0 g) were ventilated for 8 hours using rodent ventilators (FlexiVent Scireq, Montreal, PQ). After rats were anesthetized by i.p. injection of 30 mg kg-1 pentobarbital, a tracheotomy was performed. The trachea was cannulated with a 1 cm 22G cannula. Dynamic compliance was estimated from data obtained during a single-frequency forced oscillation manoeuvre, using a mathematical model-fitting technique according to the specifications of Scireq Inc. (Montreal, PQ). To determine ventilator settings we started with the normal breathing frequency of a 8-day old rat (~160 minute-1 (15)) and adjusted V_T and PEEP to achieve normal blood gases. The V_T and PEEP values for this frequency were ~12.5 ml.kg-1and 2 cm H_2O, respectively. Next we choose a lower and higher V_T and adjusted the ventilator frequency accordingly. Increasing the positive end expiratory pressure (PEEP) in the low LV_T group led to increase of CO_2 and early death, most likely due to inadvertent PEEP. Animals were monitored by ECG. Rectal temperature was maintained around 37°C by using a thermal blanket, lamp and plastic wrap. To prevent spontaneous respiratory efforts 5 $mg.kg^{-1}$ pancuronium was administered *i.p*. Every two hours 0.1 ml saline was administered to prevent dehydration. At the end of the ventilation period a blood sample from the carotid artery was taken for blood gas analysis prior to euthanasia. Lung tissues were processed for histology or fresh frozen for molecular/protein analyses. The study was conducted according to the guidelines of the Canadian Council for Animal Care and with approval of the Animal Care Review Committee of the Hospital for Sick Children.

Series I: Different Tidal Volumes. Animals were randomly assigned to one of the following four groups: 1) Non-ventilated (NV) controls; 2) Low V_T (V_T ~ 3.5 $ml.kg^{-1}$, freq. 600 $minute^{-1}$, PEEP 0 cm H_2O); 3) Moderate V_T (V_T ~12.5 $ml.kg^{-1}$, freq. 160 $minute^{-1}$, PEEP 2 cm H_2O); 4) High V_T (V_T ~ 25 $ml.kg^{-1}$, freq. 20 $minute^{-1}$, PEEP 2 cm H_2O).

Series II: Pre-exposure to LPS and Low V_T Ventilation with Oxygen. Rats were randomly assigned to injection (*i.p.*) of either 3 $mg.kg^{-1}$ body weight of LPS from *E. coli* serotype 026:B6 or the same volume of 0.9% NaCl (7). Twenty four hours after treatment animals were randomly assigned to one of the following six groups: 1) Non-ventilated after NaCl injection (NV); 2) NV after LPS injection (NV+LPS); 3) Low V_T (V_T ~ 3.5 $ml.kg^{-1}$, freq. 600 $minute^{-1}$, PEEP 0 cm H_2O) with room air after NaCl injection (LV_T): 4) Low V_T with room air after LPS injection (LV_T + LPS); 5) Low V_T with 50% oxygen after NaCl injection (LV_T+O_2); 6) Low V_T with 50% oxygen after LPS injection (LV_T+LPS/O_2).

Immunohistochemistry: After flushing lungs were infused in situ with 4% (v/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) with a constant pressure of 20 cm H_2O to equalize filling pressure over the entire lung. Under these constant pressure conditions the cannula was removed and the trachea immediately ligated. The excised lung tissue was immersed in 4% (v/v) PFA in PBS overnight and then dehydrated in an ethanol/xylene series and embedded in paraffin. Five micron sections were deparaffinized, rehydrated in a graded series of ethanol. Following antigen retrieval by heating in 10 mM sodium citrate pH 6.0, endogenous peroxidase quenching and blocking with NGS/BSA, sections were stained with 1:200 diluted mouse anti-CD68 (Serotec, Raleigh, USA) and 1: 100 diluted rabbit anti-myeloperoxidase (MPO) antibodies (Lab Vision Corporation, Fremont, Canada), using the avidin-biotin (ABC) immunoperoxidase method. Biotinylated rabbit anti-mouse IgG or goat anti-rabbit IgG were used as secondary antibodies, respectively. All sections were counterstained with hematoxylin.

Quantitative RT-PCR: Total RNA was extracted from lung tissues and reverse transcribed. Complementary DNA was amplified for our target genes (IL-1β, IL-6, IL-10, CXCL-2 (GRO2/MIP-2: macrophage inflammatory protein-2, a functional rodent homolog of human IL-8) and 18S as previously described (7, 10). For relative quantification, polymerase chain reaction signals were compared between groups after normalization using 18S as an internal reference. Fold change was calculated.

Cytokine protein measurement in BALF: Lungs were infused with 0.5 ml of saline, followed by withdrawal and re-infusion two more times (7). Total protein was determined and IL-1β, IL-6 and CXCL-1 (also known as GRO1/KC) were measured in BALF using multiplex immunoassays for Luminex technology (7). CXCL-1 was measured because of lack of CXCL-2 detection kit for the Luminex system.

Statistical analysis: Stated otherwise all data are presented as mean ± SD. Data were analyzed, using SPSS software version 15 (SPSS Inc, Chicago, IL). Depending on the distribution and the homogeneity of variation within the groups, statistical significance ($p < 0.05$) was determined by using either one-way ANOVA, or Kruskal-Wallis test. *Post hoc* analysis was performed using Duncan's multiple-range test (data presented as mean ± SD) or Mann-Whitney test (data presented as median and interquartile range). Since data of NV and LV_T groups of series I and II were similar, they were combined in the analysis.

RESULTS

Series I: Different Tidal Volumes

Physiologic data: Blood gases were in the normal range after eight hours of ventilation with different tidal volumes (Table 1). Ventilator set tidal volumes differed from inspired tidal volumes, namely 6, 16 and 40 ml.kg^{-1} for low, moderate and high $V_{T,}$ respectively.

Table 1. Blood gas analysis after 8 hours of mechanical ventilation

	Tidal Volume		
	LV_T (n=4)	MV_T (n=4)	HV_T (n=4)
pH	7.39 ± 0.08	7.38 ± 0.02	7.37 ± 0.04
$PaCO_2$(mmHg)	45.0 ± 4.4	44 ± 6.4	42 ± 1.1
PaO_2(mmHg)	72.0 ± 13.8	83 ± 8.1	91.0 ± 9.8
Saturation (%)	95.0 ± 3.2	96.0 ± 0.8	97.0 ± 1.2

LV_T = low tidal volume;
MV_T = moderate tidal volume
HV_T = high tidal volume

Dynamic compliance of the respiratory system is shown in Figure 1. Dynamic compliance of animals ventilated with high V_T significantly increased within minutes of ventilation and then remained stable for the rest of the experiment, indicative of larger airspaces and loss of tissue recoil due to hyperinflation (Fig. 2). Overall mortality during ventilation was 16.4% with no differences between V_T groups. No autopsy was performed and electrolytes were not measured.

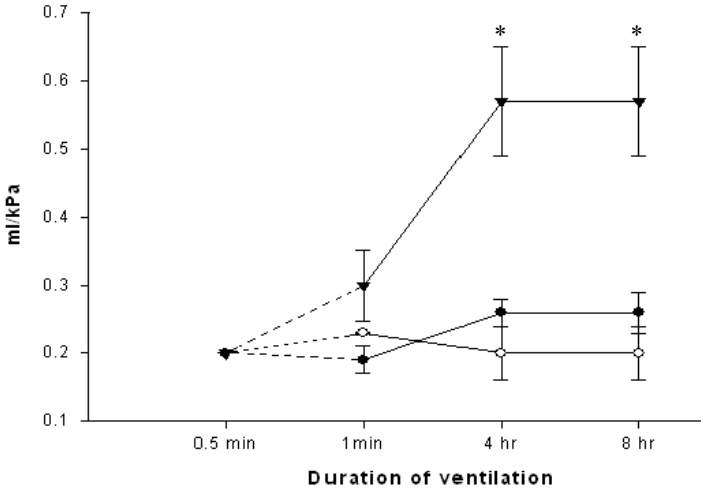


Figure 1. Dynamic compliance of 8-day newborn rats (n=8) ventilated with room air and low V_T (○), moderate V_T (●) and high V_T (▼). Dash lines are extrapolated. *$p < 0.05$ *vs*. ventilation for 1 minute.

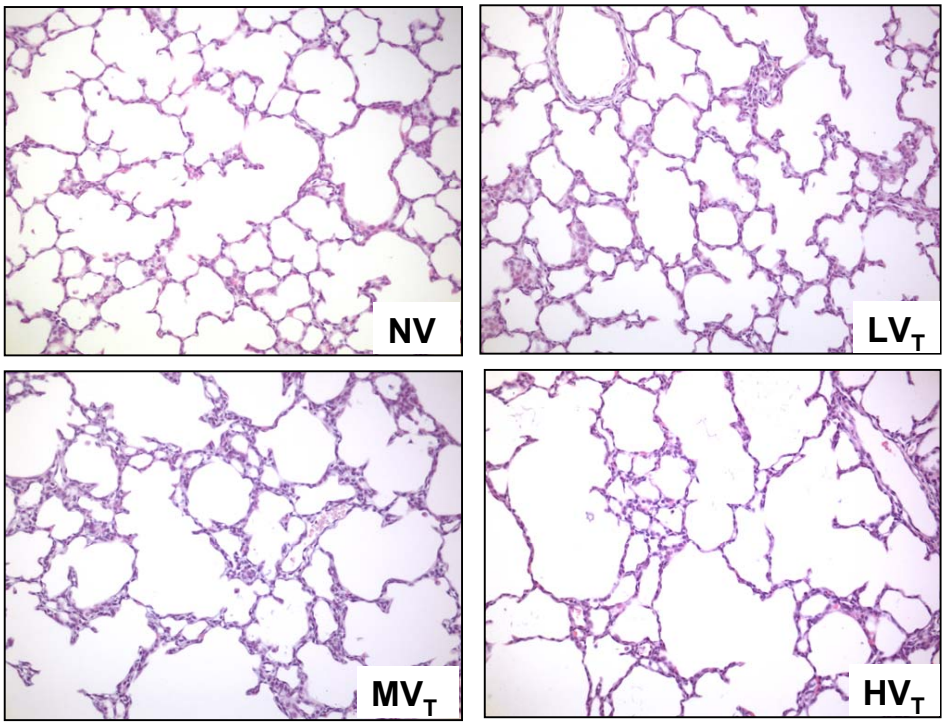


Figure 2. Representative sections of ventilated lungs stained with haematoxylin and eosin. Low, moderate and high V_T ventilation of 8-day neonatal rats do not show any areas of atelectasis, indicating no differences in lung recruitment. Air spaces are larger after high V_T ventilation due to hyperinflation.

Inflammatory cells in lung: High V_T ventilation was associated with a significant increase of MPO-positive neutrophils in comparison with NV, LV_T and MV_T. To a lesser extent, moderate V_T ventilation also increased the number of MPO-positive neutrophils (mainly in the alveolar space) in comparison with LV_T (Table 2). HV_T increased the number of neutrophils in both lung parenchyma and alveolar space. The number of macrophages (CD-68 antigen) did not alter among the ventilation groups (Table 2).

Cytokine mRNA expression: The effect of ventilation with different V_T on IL-1β, IL-6, CXCL-2, and IL-10 mRNA expression is shown in Figure 3. Low V_T ventilation increased CXCL-2 and IL-6 mRNA levels *vs*. non-ventilated animals ($p < 0.05$), while those of IL-1β and IL-10 were not altered. Moderate V_T ventilation appeared to further increase the expression of CXCL-2 *vs*. non-ventilated animals and that of IL-6 versus non-ventilated and low V_T rat pups, but the differences were not significant. Message levels of IL-1β and IL-10 were also not altered by MV_T. High V_T ventilation significantly increased mRNA expression of IL-1β, CXCL-2 and IL-6, but not IL-10, versus all other groups ($p < 0.05$).

Table 2. Effect of low, moderate and high V_T ventilation on number of myeloperoxidase- and CD68-positive inflammatory cells in lungs of of 8-day newborn rats

	Tidal Volume			
	NV	LV_T	MV_T	HV_T
MPO total	19.6 ± 8.4	14.0 ± 5.6	28.0 ± 3.0**	58.3 ± 4.2§
MPO tissue	9.7 ± 3.5	10.8 ± 6.3	8.3 ± 0.3	32.3 ± 13.1§
MPO air	10.0 ± 5.6	3.3 ± 2.8	19.7 ± 3.3*	26.0 ± 11.1*
CD 68	4.9 ± 0.9	6.1 ± 0.88	3.7 ± 0.4	4.4 ± 1.0

Number of immunopositive cells per unit area (40X high power field); n = 5 fields per slide, 3 slides per animal and 4 animals per group.
*p < 0.05 *vs.* NV and LV_T
**p < 0.05 *vs.* LV_T and HV_T
§p < 0.05 *vs.* other groups.

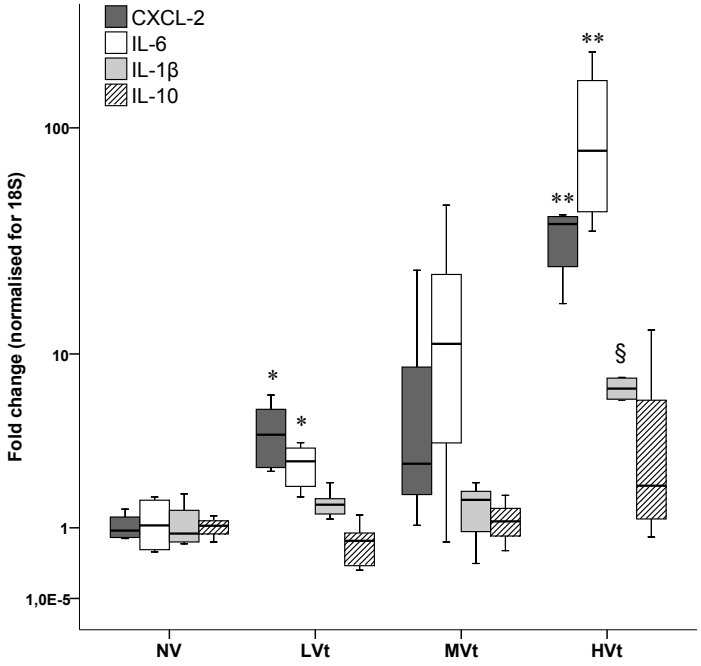


Figure 3. Effect of low, moderate and high V_T ventilation on pro-inflammatory gene expression in lungs of 8-day newborn rats. NV and LV_T: n = 8; MV_T: n = 6; HV_T: n = 4.
*p < 0.05 *vs.* non-ventilated group
**p < 0.05 *vs.* NV and LV_T group
§p < 0.05 *vs.* other groups.

Cytokines in BALF: Table 3 shows the amount of IL-1β, IL-6 and CXCL-1 in BALF after eight hours of ventilation. The volume of lavaged material recovered from each animal (0.29 ± 0.08 ml) and BALF total protein content (0.22 ± 0.08 μg/μl) did not differ

Table 3. Effect of low, moderate and high V_T ventilation on BALF cytokine protein content of 8-day newborn rats

Cytokine	NV (n=8)	LV_T (n=4)	MV_T (n=4)	HV_T (n=4)
IL-6	0.7 (0.6-1.1)	2.2 (1.1.-4.9)	8.0 (3.3-11.7)	5.2 (1.7-21.0)
IL-1β	0.13 (0.1-0.2)	0.2 (0.17-0.4)	0.33 (0.24-0.43)	1.0 (0.94-1.5)*
GRO/KC	10.3 (7.5-12.3)	9 (8.5-9.8)	7.9 (3.7-12.0)	14.9 (12.3-20.7)**

Concentrations are expressed as pg cytokine/100 pg of total BALF protein.
*$p < 0.05$ *vs*. NV, LV_T and MV_T
**$p < 0.05$ vs. LV_T and MV_T.

significantly between treatment groups. There was a trend towards an increase of IL-1β and IL-6 with increasing tidal volume. However, only HV_T ventilation significantly increased IL-1β and CXCL-1 levels.

Series II: Pre-exposure to LPS and Low Tidal Volume Ventilation with Oxygen

Physiologic data: Ventilation for 8 hours with low V_T with room air after exposure to LPS (LV_T+LPS), ventilation with 50% oxygen (LV_T+O_2), and ventilation with oxygen after exposure to LPS (LV_T+LPS/O_2) resulted in normal pH and $PaCO_2$ (Table 4). Low V_T ventilation with room air after exposure to LPS (LV_T +LPS) and ventilation with 50% oxygen (LV_T+O_2) significantly increased the PaO_2 when compared to ventilation with room air (LV_T). The combination of ventilation with oxygen and exposure to LPS (LV_T+LPS/O_2) further increased PaO_2 *vs*. LV_T and LV_T+LPS groups ($p < 0.05$). Mean airway pressures and peak pressures remained stable during the ventilation period and were not different between groups.

Table 4. Blood gas analysis after 8 hours of mechanical ventilation of 8-day newborn rats after exposure to endotoxin and/or 50% oxygen

	Tidal Volume		
	LV_T + LPS	$LV_T + O_2$	LV_T + LPS/ O_2
pH	7.40 ± 0.04	7.32 ± 0.18	7.30 ± 0.08
$PaCO_2$(mmHg)	36.0 ± 3.7	58.3 ± 14.4	54.3 ±16.6
PaO_2(mmHg)	87.7 ± 6.6*	131.0 ± 52.1*	167.0 ± 40.4**
Saturation (%)	96.5 ± 1.2	96.4 ± 3.6	98.4 ± 1.6

*$p < 0.05$ versus animals ventilated with LV_T
**$p < 0.05$ versus animals ventilated with LV_T and LV_T + LPS.

Dynamic compliance of the respiratory system is shown in Figure 4. Ventilation with room air after exposure to LPS (LV_T+LPS), ventilation with 50% oxygen (LV_T+O_2), and ventilation with oxygen after exposure to LPS (LV_T+LPS/O_2) significantly decreased the dynamic compliance after 4 hours of ventilation. Loss of compliance can be explained by increase of stiffness of the lung as a result of lung injury. No further worsening of dynamic compliance was seen during the last 4 hours of ventilation. Overall mortality

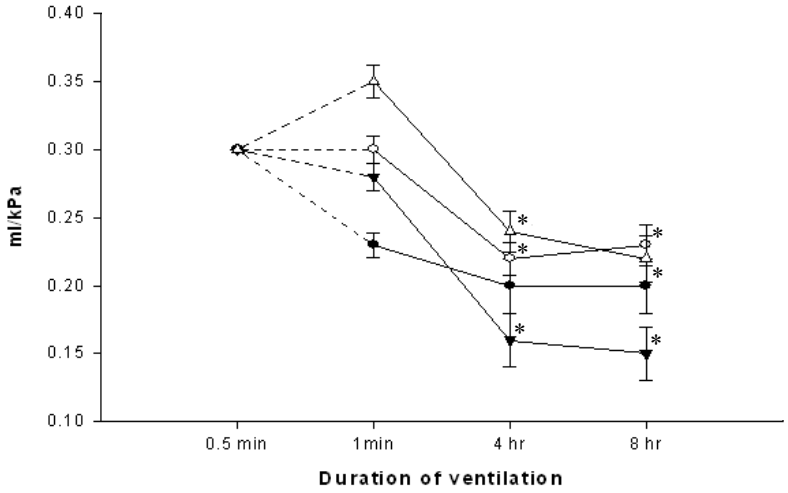


Figure 4. Dynamic compliance of 8-day newborn rats ventilated with low V_T and room air (●), low V_T and room air after exposure to LPS (○), low V_T and 50% oxygen (▼) and low V_T with 50% oxygen after exposure to LPS (▽). n = 8 animals per group. The dash lines are extrapolated. *p <0.05 versus ventilation for 1 minute.

during ventilation was 8.1% with no differences between the 4 groups. No autopsy was performed.

Inflammatory cells in lung: Mean values and ranges for the number of macrophages (CD68- antigen) and MPO-positive neutrophils per unit area are shown in Table 5. Exposure to LPS, independent of ventilation with or without oxygen, was associated with a significant increase of MPO-positive neutrophils as well as CD-68 positive macrophages. The number of neutrophils was profoundly increased in the parenchyma and to a lesser extent in the alveolar space.

Cytokine mRNA expression: The effect of LPS, LV_T ventilation and LV_T ventilation after exposure to LPS (LV_T+LPS) on IL-1β, IL-6, CXCL-2 and IL-10 mRNA expression is shown in Figure 5A. LPS significantly increased the expression of IL-6 and IL-1β even 24 hours after administration when compared to saline treated animals (LPS>NV, $p < 0.05$). However, mRNA expression of CXCL-2 and IL-10 was not altered by the LPS pre-treatment. The combination of LV_T and LPS pretreatment did not increase the expression of IL-6 and CXCL-2 above that observed in animals ventilated with LV_T ($LV_T \approx LV_T$+LPS>NV; $p < 0.05$) and decreased IL-6 mRNA levels compared to LPS treatment alone (LPS>LV_T+LPS, $p < 0.05$). LV_T ventilation after exposure to LPS significantly decreased the expression of IL-10 mRNA ($p < 0.05$). Figure 5B shows the effect of ventilation with room air or 50% oxygen on IL-1β, IL-6, CXCL-2 and IL-10 mRNA expression. As shown in Series I, low V_T ventilation with room air increased the expression of IL-6 and CXCL-2 *vs.* non-

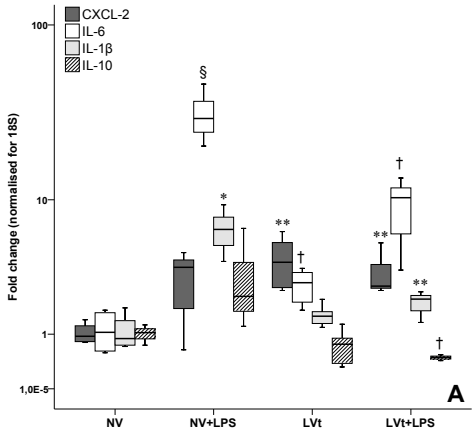
Table 5. Combined effect of endotoxin, oxygen and low V_T ventilation on number of myeloperoxidase- and CD68-positive inflammatory cells in lungs of 8-day newborn rats

	Tidal Volume		
	NV	NV+LPS	LV_T
MPO total	19.6 ± 8.4	211.0 ± 74.0**	14.0 ± 5.6
MPO tissue	9.7 ± 3.5	187.3 ± 73.3**	10.8 ± 6.3
MPO air	10.0 ± 5.6	23.8 ± 2.2†	3.3 ± 2.8
CD 68	4.9 ± 0.9	11.8 ± 0.33**	6.1 ± 0.88
	LV_T+LPS	LV_T+O_2	LV_T+LPS/O_2
MPO total	186.0 ± 117.5**	12.0 ± 1.4	188.7 ± 257.9§
MPO tissue	169.3 ± 114.3**	8.3 ± 2.9	176.8 ± 259.3§
MPO air	16.7 ± 9.7*	3.7 ± 3.5	11.8 ± 3.4**
CD 68	2.5 ± 2.8**	7.4 ± 0.6	13.4 ± 2.0**

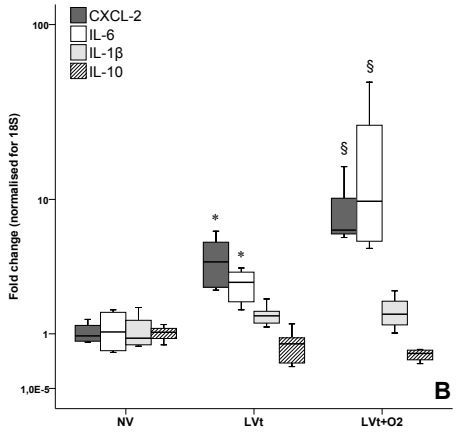
Number of immunopositive cells per unit area (40X high power field). LV_T+O_2 group was not compared with NV+LPS and LV_T+LPS groups; n = 5 fields per slide, 3 slides per animal and 4 animals per group.
*$p < 0.05$ *vs.* LV_T
**$p < 0.05$ *vs.* NV and LV_T
§$p < 0.05$ *vs.* NV, LV_T and LV_T+O_2
†$p < 0.05$ *vs.* NV, LV_T and LVT+LPS/O_2

ventilated controls. Ventilation with 50% oxygen further increased the message levels of both cytokines ($LV_T+O_2>LV_T$, $p < 0.05$). The expression of IL-1β and IL-10 mRNA was not altered by ventilation with room air or oxygen. Ventilation with 50% O_2 after LPS pre-treatment (Fig. 5C) resulted in the greatest increase in IL-6 mRNA levels (LV_T+LPS/O_2>NV+LPS>LV_T+LPS≈$LV_T+O_2>LV_T$>NV) (Fig. 5A-C). Interestingly, the combination of ventilation with oxygen and pre-exposure to LPS decreased the expression of CXCL-2 *vs*. ventilation with room air (LV_T+LPS/O_2≈NV<LV_T, $p < 0.05$) (Fig. 5C).

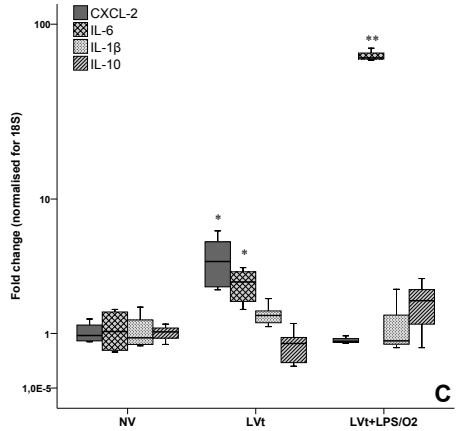
Cytokines in BALF: Table 6 shows the amount of IL-1β, IL-6 and CXCL-1 protein in BALF after exposure to either LPS, ventilation with room air or oxygen, ventilation after exposure to LPS or the combination of ventilation with oxygen after exposure to LPS. The amount of BALF total protein was significantly increased after exposure to LPS and further increased after ventilation with room air or oxygen and after ventilation with oxygen of a LPS exposed lung (LV_T+LPSLO2≈LV_T+O_2≈LV_T+LPS≈LV_T>LPS>NV, $p < 0.05$), consistent with lung injury (Fig. 6). The volume of lavaged material recovered from each animal (0.31 ± 0.06 ml) did not differ significantly between the groups. IL-6 content of BALF increased after ventilation with oxygen (LV_T+O_2>NV, $p<0.05$). An increase was also noted after ventilation of a LPS-exposed lung (LV_T+LPS>NV≈NV+LPS, $p < 0.05$). Ventilation of a LPS exposed lung with oxygen did not further increase the BALF IL-6



Low V_T after exposure to LPS
*p < 0.05 *vs.* NV and LV_T
**p < 0.05 *vs.* NV,
§p <0.05 *vs.* NV, LV_T and LV_T+LPS
†p < 0.05 *vs.* NV and NV +LPS



Low V_T ventilation with 50% oxygen
*p < 0.05 *vs.* NV and LVt+O_2
§p < 0.05 *vs.* NV and V_T



Low V_T ventilation with 50% oxygen after exposure to LPS
*p < 0.05 *vs.* NV and LV_T +LPS/O_2
**p < 0.05 *vs.* NV and LV_T

Figure 5. Effect of endotoxin and oxygen on low V_T ventilation triggered pro-inflammatory gene expression in lungs of 8-day newborn rats. NV and LV_T: n = 8; NV+LPS, LV_T +O_2 and LV_T +LPS: n = 5; LV_T +LPS/O_2: n = 6.

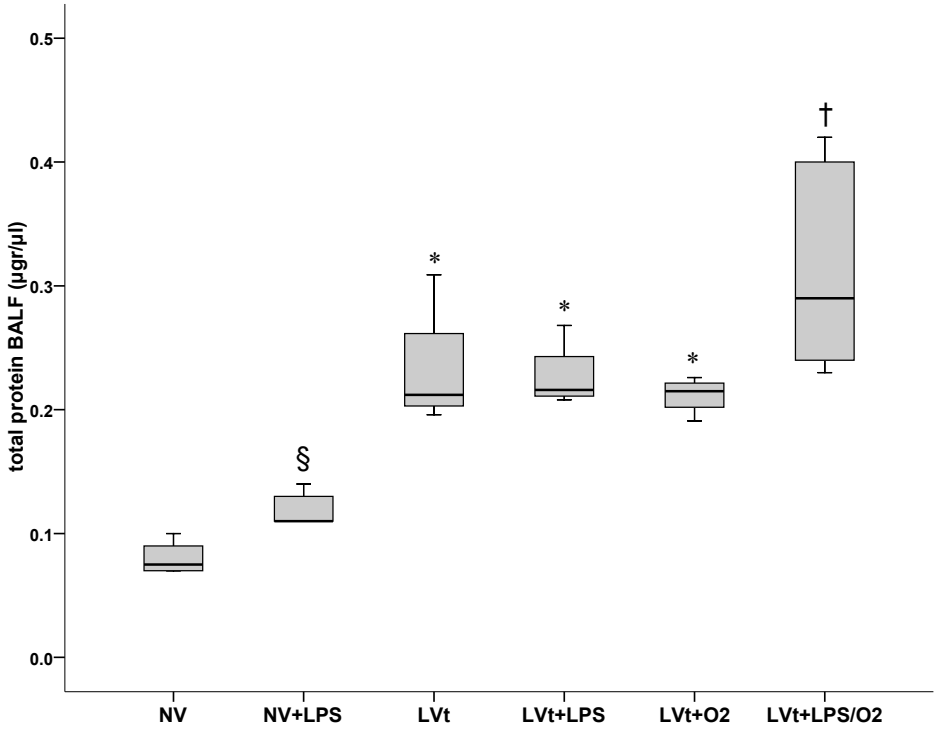


Figure 6. Effects of low V_T ventilation, endotoxin and oxygen on BALF total protein of 8-day newborn rats. NV and LV_T: n = 8; NV+LPS: n = 5; LV_T+LPS and LV_T+O_2: n = 4; LV_T+LPS/O_2: n = 6. $LV_T + O_2$ group was not compared with NV+LPS and LV_T+LPS groups.
*p<0.05 *vs*. NV
§p<0.05 *vs*. LV_T, LV_T+LPS and LV_T+LPS/O_2,
†p<0.05 *vs*. LV_T+LPS and LV_T+O_2.

content (LV_T+LPS/$O_2 \approx LV_T+O_2 \approx LV_T$+LPS, $p > 0.05$). The concentration of IL-1β in BALF only increased after ventilation with oxygen and after ventilation with oxygen after LPS exposure (LV_T+LPS/$O_2 \approx LV_T+O_2 > LV_T$+LPS$\approx LV_T \approx$NV, $p < 0.05$). Independent of ventilation and oxygen, the concentration of CXCL-1 was increased after exposure to LPS (NV +LPS$\approx LV_T$+LPS$> LV_T \approx LV_T+O_2 \approx LV_T$+LPS/$O_2$, $p > 0.05$).

Table 6. Combined effect of endotoxin, oxygen and low V_T ventilation on BALF cytokine protein content of 8-day newborn rats

Cytokine	NV	NV+LPS	LV_T
IL-6	0.7 (0.6-1.1)	0.7 (0.5-1.1)	2.2 (1.1.-4.9)
IL-1β	0.13 (0.1-0.2)	0.07 (0.06-0.08)§	0.2 (0.17-0.4)
GRO/KC	10.3 (7.5-12.3)	22.2 (18.3-23.4)†	9 (8.5-9.8)
	LV_T+LPS	LV_T+O_2	LV_T+LPS/O_2
IL-6	3.5 (2.6-3.8)**	2.1 (1.3-2.9)*	3.0 ± 0.68**
IL-1β	0.22 (0.18-0.23)	0.45 (0.38-0.55)*	0.29 (0.25-0.45)¶
GRO/KC	15.7 (10-21.4)‡	7.6 (7.0-9.2)	9.6 (7.4-12.2)

Concentrations are expressed as pg cytokine/100 pg of total BALF protein. n=8 for groups NV and LV_T, n = 4 animals for other groups. LV_T+O_2 group was not compared with NV+LPS and LV_T+LPS groups.
§$p < 0.05$ *vs*. NV, LV_T, LV_T+LPS and LV_T+LPS/O_2
†$p < 0.05$ *vs*. NV, LV_T;
‡$p < 0.05$ *vs*. NV, LV_T and LV_T+LPS/O_2
¶$p < 0.05$ *vs*. NV, NV+LPS and LV_T+LPS
*$p < 0.05$ *vs*. NV
**$p < 0.05$ *vs*. NV and NV+LPS

DISCUSSION

Mechanical ventilation, (intrauterine) infection and oxygen are well-recognized risk factors for BPD and known to trigger a pro-inflammatory response. In the present study we demonstrate that low V_T ventilation -presumed to be a less injurious form of ventilation- triggers a pro-inflammatory cytokine/chemokine response in neonatal rats, which is amplified by ventilation with oxygen, but not endotoxin pre-treatment. The combination of ventilation with oxygen and endotoxin pre-treatment either intensifies or abrogates the low V_T response, depending on individual cytokine or chemokine.

In the first series of experiments we assessed the effect of low, moderate, and high V_T ventilation on pro- and anti-inflammatory cytokine/chemokine production. Clinical data and experimental studies using premature animal models have compared high frequency oscillatory ventilation (HFOV: low V_T) and conventional ventilation (CMV: high V_T) with respect to pro-inflammatory cytokine/chemokine production and release into the alveolar and/or vascular compartment. Some studies reported findings favouring low V_T ventilation (4, 16) whereas others did not find any significant differences in cytokine/chemokine production/release between CMV and HFOV ventilation (17, 18). In the present study, we found a significant increase of mRNA expression of CXCL-2 and IL-6 after 8 hours of low V_T ventilation. Further increases were noted with increasing V_T as reported previously (14). Although there was a tendency to higher concentrations of IL-6 and IL-1β protein in the BAL fluid, we did not find significant increases in total

number of inflammatory cells after low V_T ventilation. In contrast, moderate and to a higher extent high V_T ventilation increased the inflammatory response as shown by increases in IL-6, IL-1β and CXCL-1 content in BALF and number of inflammatory cells in the lung. In addition, high V_T altered the dynamic compliance due to hyperinflation as shown previously (7). The difference between set and inspired V_T suggests tube leakage and/or expansion of tubing of the ventilator circuit which may influence the compliance measurement. No clinical signs for pneumothorax were observed. Thus, low V_T ventilation with room air for 8 hours results in a mild inflammatory response in the neonatal lung that is not overtly injurious (no change in dynamic compliance but an increase in the amount of protein in BALF). However, it is plausible that longer durations of low V_T ventilation increase the levels of pro-inflammatory cytokines sufficiently to cause lung injury. Although the low V_T ventilation strategy (~ 3.5 $ml.kg^{-1}$) cannot be compared with clinical applied HFOV (V_T 0.5-2.0 $ml.kg^{-1}$), our finding of an inflammatory response may explain why randomised controlled trials did not show any beneficial effect of protective HFOV in preventing BPD in premature infants. This explanation is supported by several studies in which MV elevates pulmonary cytokines without cellular injury (19-21).

LPS triggers a network of inflammatory responses by activation of macrophages and recruitment of neutrophils, which was also observed in the present study. Activated macrophages release different pro-inflammatory cytokines and neutrophil activation causes the production of oxygen radicals and the release of granular enzymes, which are associated with injurious processes in the lung (10, 22, 23). Especially CXC chemokines and IL-8 activate and attract neutrophils into interstitial and alveolar spaces of the lung. Blocking neutrophils by blocking the CXCL-2 receptor led to increased alveolar formation and CXCL-2 null mice exhibited less ventilator-induced lung injury (10, 24). Several studies have shown that high V_T ventilation combined with another lung injury amplifies the inflammatory response in adult lungs (25, 26). A significant increase of CXCL-2 was measured in BALF of adult rats when high V_T ventilation (40 $ml.kg^{-1}$) was superimposed on a systemic inflammatory process (25). Ventilation of adult mice with smaller tidal volumes (6 $ml.kg^{-1}$) after induction of lung injury with hydrochloric acid showed a significant increase of IL-6 content in lung tissue versus ventilation alone (26). High V_T ventilation of neonatal rat lungs superimposed on a systemic inflammation induced by LPS (7) significantly increased IL-6 mRNA expression compared to high V_T ventilation alone. In the present study, we found an additive effect of 50% oxygen on LV_T-induced expression of IL-6, in agreement with our previous study using 100% oxygen and high V_T (14). Whether the additive effect of oxygen on IL-6 expression is harmful remains a matter of speculation. IL-6 has long been considered a pro-inflammatory cytokine but adult transgenic mice that over-express IL-6 are more resistant to oxidative injury (27), while newborn IL-6 transgenic mice demonstrated more cell death after 100% oxygen

exposure (28). These data suggest that high levels of IL-6 in the lung may actually be beneficial in adult mice, but harmful in newborn mice. Surprisingly, we found that pre-exposure to LPS did not further increase IL-6 message levels when compared to LV_T ventilation alone. In contrast, the combination of low V_T ventilation with 50% oxygen after exposure to LPS further amplified the mRNA expression of IL-6. The increased expression of IL-6 mRNA after LV_T ventilation with and without oxygen, which was reflected in increased concentrations of this cytokine in BALF. LPS pre-exposure did not further increase BALF levels of IL-6. LV_T ventilation with 50% oxygen also increased the mRNA expression of CXCL-2 versus ventilation alone. Pre-treatment with LPS had no significant additive effect on CXCL-2 expression, in contrast to our previous findings with high V_T ventilation (7). No correlation between CXCL-2 mRNA expression and CXCL-1 protein content in BALF was observed, suggesting that CXCL-2 and CXCL-1 are not interchangeable. Together, these findings suggest that low V_T ventilation avoids the synergistic effect of ventilation and systemic inflammation on cytokine/chemokine expression seen with high V_T (7, 10). In contrast, oxygen has an additive effect on ventilation-induced cytokine/chemokine expression, which is tidal volume independent. Interestingly, upregulated CXCL-2 message was reduced to non-ventilated control levels when LPS-treated newborn rats were ventilated with 50% oxygen. This complex immunomodulatory regulation of CXCL-2 and IL-6 resembles that seen in LPS tolerant mice (29). Pulmonary IL-6 levels were significantly increased in the tolerant mice upon further LPS challenge, while CXCL-2 levels were significantly reduced. However, it is unlikely that a single exposure to LPS induced tolerance in our model.

We conclude that even low tidal volume ventilation can mount an inflammatory response in the newborn rat, which is amplified by a clinically relevant concentration of inspired oxygen.

REFERENCES

1. Speer CP 2006 Inflammation and bronchopulmonary dysplasia: a continuing story. Semin Fetal Neonatal Med 11:354-362
2. Ghezzi F, Gomez R, Romero R, Yoon BH, Edwin SS, David C, Janisse J, Mazor M 1998 Elevated interleukin-8 concentrations in amniotic fluid of mothers whose neonates subsequently develop bronchopulmonary dysplasia. Eur J Obstet Gynecol Reprod Biol 78:5-10
3. Groneck P, Gotze-Speer B, Oppermann M, Eiffert H, Speer CP 1994 Association of pulmonary inflammation and increased microvascular permeability during the development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluids of high-risk preterm neonates. Pediatrics 93:712-718
4. Yoder BA, Siler-Khodr T, Winter VT, Coalson JJ 2000 High-frequency oscillatory ventilation: effects on lung function, mechanics, and airway cytokines in the immature baboon model for neonatal chronic lung disease. Am J Respir Crit Care Med 162:1867-1876
5. Imai Y, Nakagawa S, Ito Y, Kawano T, Slutsky AS, Miyasaka K 2001 Comparison of lung protection strategies using conventional and high-frequency oscillatory ventilation. J Appl Physiol 91:1836-1844
6. Altemeier WA, Matute-Bello G, Frevert CW, Kawata Y, Kajikawa O, Martin TR, Glenny RW 2004 Mechanical ventilation with moderate tidal volumes synergistically increases lung cytokine response to systemic endotoxin. Am J Physiol Lung Cell Mol Physiol 287: L533-L542
7. Roth-Kleiner M, Ridsdale R, Cao L, Kuliszewski M, Tseu I, McKerlie C, Post M 2007 Lipopolysaccharide exposure modifies high tidal volume ventilation-induced proinflammatory mediator expression in newborn rat lungs. Pediatr Res 61:191-196
8. Bonikos DS, Bensch KG, Northway WH, Jr., Edwards DK 1976 Bronchopulmonary dysplasia: the pulmonary pathologic sequel of necrotizing bronchiolitis and pulmonary fibrosis. Hum Pathol 7:643-666
9. Jankov RP, Johnstone L, Luo X, Robinson BH, Tanswell AK 2003 Macrophages as a major source of oxygen radicals in the hyperoxic newborn rat lung. Free Radic Biol Med 35: 200-209
10. Yi M, Jankov RP, Belcastro R, Humes D, Copland I, Shek S, Sweezey NB, Post M, Albertine KH, Auten RL, Tanswell AK 2004 Opposing effects of 60% oxygen and neutrophil influx on alveologenesis in the neonatal rat. Am J Respir Crit Care Med 170:1188-1196
11. Davis JM, Dickerson B, Metlay L, Penney DP 1991 Differential effects of oxygen and barotrauma on lung injury in the neonatal piglet. Pediatr Pulmonol 10:157-163
12. Delemos RA, Coalson JJ, Gerstmann DR, Kuehl TJ, Null DM, Jr. 1987 Oxygen toxicity in the premature baboon with hyaline membrane disease. Am Rev Respir Dis 136:677-682
13. Copland IB, Kavanagh BP, Engelberts D, McKerlie C, Belik J, Post M 2003 Early changes in lung gene expression due to high tidal volume. Am J Respir Crit Care Med 168:1051-1059
14. Copland IB, Martinez F, Kavanagh BP, Engelberts D, McKerlie C, Belik J, Post M 2004 High tidal volume ventilation causes different inflammatory responses in newborn versus adult lung. Am J Respir Crit Care Med 169:739-748
15. Liu Q, Lowry TF, Wong-Riley MT 2006 Postnatal changes in ventilation during normoxia and acute hypoxia in the rat: implication for a sensitive period. J Physiol 577:957-970
16. Capoluongo E, Vento G, Santonocito C, Matassa PG, Vaccarella C, Giardina B, Romagnoli C, Zuppi C, Ameglio F 2005 Comparison of serum levels of seven cytokines in premature

newborns undergoing different ventilatory procedures: high frequency oscillatory ventilation or synchronized intermittent mandatory ventilation. Eur Cytokine Netw 16:199-205
17. Vento G, Matassa PG, Ameglio F, Capoluongo E, Zecca E, Tortorolo L, Martelli M, Romagnoli C 2005 HFOV in premature neonates: effects on pulmonary mechanics and epithelial lining fluid cytokines. A randomized controlled trial. Intensive Care Med 31:463-470
18. Thome U, Gotze-Speer B, Speer CP, Pohlandt F 1998 Comparison of pulmonary inflammatory mediators in preterm infants treated with intermittent positive pressure ventilation or high frequency oscillatory ventilation. Pediatr Res 44:330-337
19. Vaneker M, Halbertsma FJ, van Egmond J, Netea MG, Dijkman HB, Snijdelaar DG, Joosten LA, van der Hoeven JG, Scheffer GJ 2007 Mechanical ventilation in healthy mice induces reversible pulmonary and systemic cytokine elevation with preserved alveolar integrity: an in vivo model using clinical relevant ventilation settings. Anesthesiology 107:419-426
20. Yamamoto H, Teramoto H, Uetani K, Igawa K, Shimizu E 2002 Cyclic stretch upregulates interleukin-8 and transforming growth factor-beta1 production through a protein kinase C-dependent pathway in alveolar epithelial cells. Respirology 7:103-109
21. Vlahakis NE, Schroeder MA, Limper AH, Hubmayr RD 1999 Stretch induces cytokine release by alveolar epithelial cells in vitro. Am J Physiol 277:L167-L173
22. Sibille Y, Reynolds HY 1990 Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 141:471-501
23. Jobe AH, Ikegami M 1998 Mechanisms initiating lung injury in the preterm. Early Hum Dev 53:81-94
24. Belperio JA, Keane MP, Burdick MD, Londhe V, Xue YY, Li K, Phillips RJ, Strieter RM 2002 Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury. J Clin Invest 110:1703-1716
25. Tremblay L, Valenza F, Ribeiro SP, Li J, Slutsky AS 1997 Injurious ventilatory strategies increase cytokines and c-fos m-RNA expression in an isolated rat lung model. J Clin Invest 99:944-952
26. Gurkan OU, O'Donnell C, Brower R, Ruckdeschel E, Becker PM 2003 Differential effects of mechanical ventilatory strategy on lung injury and systemic organ inflammation in mice. Am J Physiol Lung Cell Mol Physiol 285:L710-L718
27. Ward NS, Waxman AB, Homer RJ, Mantell LL, Einarsson O, Du Y, Elias JA 2000 Interleukin-6-induced protection in hyperoxic acute lung injury. Am J Respir Cell Mol Biol 22: 535-542
28. Choo-Wing R, Nedrelow JH, Homer RJ, Elias JA, Bhandari V 2007 Developmental differences in the responses of IL-6 and IL-13 transgenic mice exposed to hyperoxia. Am J Physiol Lung Cell Mol Physiol 293:L142-L150
29. Natarajan S, Kim J, Remick DG 2010 Chronic pulmonary LPS tolerance induces selective immunosuppression while maintaining the neutrophilic response. Shock 33:162-169

Chapter 4

Apoptotic Cell Death in Bronchopulmonary Dysplasia

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Current Pediatric Reviews 2011; 7(4): 285-292

ABSTRACT

Apoptosis plays an important role in normal lung development as well as repair after lung injury and contributes to the pathophysiology of many lung diseases. Bronchopulmonary dysplasia (BPD) is a major cause of neonatal pulmonary and non-pulmonary morbidity and is characterized by an arrest in alveolar development. Currently there is no specific treatment for BPD. Mechanical ventilation, exposure to high concentrations of oxygen and inflammation are important risk factors for the development of BPD. Emerging evidence links the pathophysiology of BPD to an imbalance between anti-apoptotic and pro-apoptotic signaling pathways. Different apoptotic signaling pathways have been implicated, including Fas/FasL, caspase-dependent and -independent pathways, pro-survival Akt, transforming growth factor-β and p53. To what extent these pathways are involved in the pathogenesis of BPD is a hot topic of research. The aim of this review is to describe the timing and apoptotic events during lung development and the pathogenesis of BPD with particular focus upon apoptotic pathways activated by mechanical ventilation, hyperoxia and inflammation. An appreciation of these apoptotic pathways is essential for understanding the aetiology of and the development of treatments for pulmonary diseases such as BPD.

INTRODUCTION

Apoptosis is important for the right balance between the formation of new cells by mitosis, and the orderly destruction and removal of cells that are no longer needed by the organism. It is critical for normal lung development and function (1-2). In addition, apoptosis plays a vital role in remodelling of lung tissue after injury by clearance of excess epithelial and mesenchymal cells (3-4). Dysfunctional apoptotic mechanisms appear to be important in the pathophysiology of many lung diseases, including interstitial pulmonary fibrosis, chronic obstructive pulmonary disease and acute respiratory distress syndrome (5-11). Bronchopulmonary dysplasia, most commonly defined as an ongoing requirement for supplemental oxygen and/or respiratory support at 36 weeks post-menstrual age (12), is a chronic respiratory disease that develops as a consequence of neonatal lung injury. BPD is mainly seen in very low birth weight infants and is characterized by an arrest in alveolar and vascular development (13-14). Mechanical ventilation, intra-uterine infections and oxidative stress are well-recognized risk factors for development of BPD (15-17). Patients with BPD remain oxygen dependent for many months and in the first two years they frequently require hospital readmission and use more medication than patients without BPD (18-19). The most severely affected may remain symptomatic and have evidence of airway obstruction as adults (20).

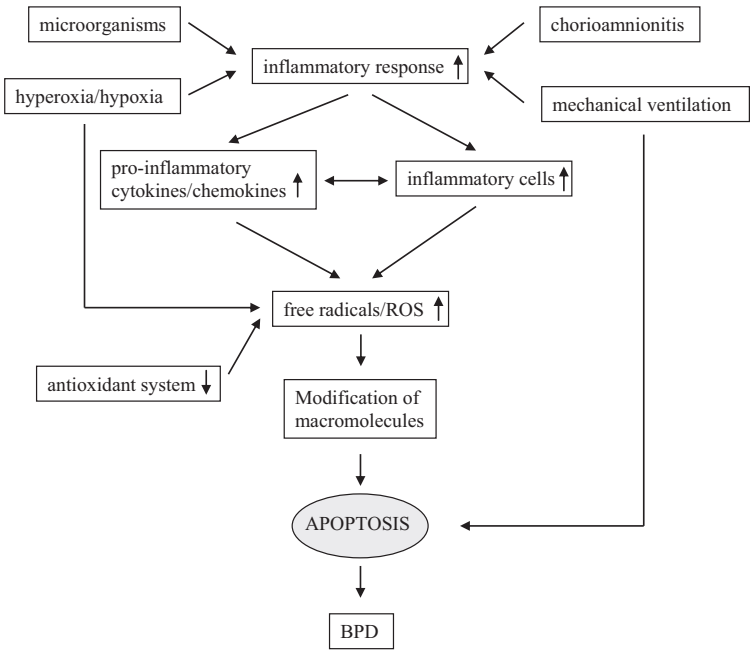

Figure 1. Possible pathogenetic sequence of events in the development of bronchopulmonary dysplasia.

The clinical treatment of preterm infants is complex. For example, mechanical ventilation is combined with oxygen treatment, making it hard to study the influence of each risk factor separately. Here we will discuss the importance of apoptosis for normal lung development and how that process can be altered by cell stretch (mechanical ventilation), hyperoxia and inflammation - the prime risk factors for BPD (Fig. 1).

Apoptotic pathways

Apoptosis is only one of several types of programmed cell death (21). Apoptotic cells are characterized by cytoplasmic and nuclear shrinkage, and the formation of apoptotic bodies. The cellular membrane of apoptotic cells remains intact (22). The result is the complete elimination of cellular debris without induction of an inflammatory response (23-24). Apoptosis is genetically determined during the coarse of development, but can also be triggered by external stimuli, such as inflammation and intrinsic stimuli, resulting from alteration of cellular function and metabolism (25). During apoptosis various proteases including caspases are activated. The human genome encodes 13 distinct caspases of which seven have been suggested to participate in apoptosis as initiator (caspase-2, -8, -9 and 10) or effector (caspase-3, -6 and 7) caspases (25-26). Caspases can be activated by either an extrinsic or intrinsic pathway (27-28). The extrinsic pathway is activated by binding of members of the tumor necrosis family (TNF) such as Fas Ligand (FasL) and TNF-α to specific cell surface death receptors including Fas receptor (CD95), TNF receptors I and II, DR4 and DR5 (29-31). Ligand binding is translated into intracellular signals that eventually leads to activation of effector caspases 3 and 7 via upstream initiator caspases 8 and 10 (27). Activated caspases 3 and 7 set off enzymes responsible for apoptosis resulting in nuclear condensation and fragmentation (26, 29). Studies have found increased concentrations of soluble Fas and FasL in the bronchoalveolar lavage (BAL) of patients with acute lung injury and acute respiratory distress syndrome (10). Excessive apoptosis of epithelial cells and infiltrating lymphocytes expressing FasL mRNA has been observed in fibrotic lung tissue of bleomycin-treated mice, which was abolished by the administration of a soluble form of Fas antigen or anti-FasL antibody (32). These findings suggest involvement of the Fas/FasL pathway in mediating apoptosis during lung injury. The intrinsic pathway is activated by physical and chemical stress signals, leading to changes in mitochondrial outer membrane integrity (26). The integrity of the mitochondrial outer membrane is determined by the balance between anti-apoptotic (e.g., Bcl-2, Bcl-$_{XL}$) and pro-apoptotic (e.g., Bax, Bad, Bak) B-cell lymphoma (Bcl-2) family proteins (29, 33). Permeabilization of the mitochondrial outer membrane will lead to release of pro-apoptotic factors such as cytochrome c. The release of these factors leads to the activation of caspase-9 and ultimately results in the activation of effector caspases including caspase-3. In certain conditions the extrinsic and intrinsic pathway are interlinked by caspase-8 (29). Besides

caspases as executioners of programmed cell death, there is increasing evidence of the existence of caspase-independent pathways leading to apoptosis (34-35). Cathepsin B, a proteolytic lysosomal enzyme, seems to be the main executor of this pathway (36). In addition, lung cells can die via oncosis and autophagy (37-41).

Apoptosis and lung development

Development of the fetal lung can be divided into six overlapping stages (42). Apoptotic activity has been observed throughout each stage (43). During the embryonic stage (weeks 4-5 in humans) a diverticulum from the foregut endoderm invades into the surrounding splanchnopleuric mesoderm. The lung anlage gives rise to lung sacs on either side of the foregut tube that begins to separate at the same time into a ventral trachea and a dorsal oesophagus. Apoptosis occurs in the peripheral mesenchyme surrounding these lung sacs and underlying airway branch points (44). The pseudoglandular stage (weeks 5-17 in humans) is characterized by branching morphogenesis, establishing the prospective pre-acinar airways (i.e. those proximal to the respiratory bronchioles) (45). Apoptosis is found in the interstitial tissue, leading to mesenchymal involution (2, 46). The subsequent transition to the canalicular stage (weeks 16-26 in humans) is marked by completion of airway branching. The terminal bud epithelium is reduced from a pseudostratified form to simple cuboidal, marking the appearance of the future acini. The epithelium begins to differentiate into alveolar type II cells. The mesenchyme thins out by apoptosis -first peak of apoptosis during lung development- and developing capillaries begin to "canalize" the lung parenchymal interstitium of the primitive respiratory units (2, 42, 46). Apoptosis at this stage of lung development maybe mediated via the Fas/FasL pathway (47). During the saccular period (weeks 24-38 in humans) distal airways expand and obtain a saccular shape. There is further thinning of the mesenchyme by apoptosis and flattening of the cuboidal alveolar type II epithelial cells into squamous alveolar type I cells, leading to a close link between the capillary network and airway epithelium (2, 46, 48-49). The alveolar period (weeks 36-8 years in humans) is characterized by the formation and subsequent extension of secondary septa, thereby subdividing the immature saccules into smaller units. The capillary network forms a double layer of capillaries in each saccular wall. During this period an excess of alveolar type II cells are produced (50-51). These cells serve as putative stem cells for alveolar type I cells and produce surfactant. An excess of alveolar type II cells guarantees that enough alveolar type II cells can be formed when the alveolar surface area increases during the alveolarization period. Moreover, an excess of alveolar type II cells ensures enough pulmonary surfactant around birth (51). After birth excess of alveolar type II cells and fibroblasts are removed by apoptosis; second peak of apoptosis during lung development (2, 46, 50-51). The expression of p53 is up-regulated during this period in a time-dependent manner, indicating that a

p53-dependent pathway may be involved in regulating apoptosis at this time (52). Cell cycle regulator p53 is a transcriptional factor that can either arrest the cell cycle for repair of damaged DNA or initiate apoptotic cell death (53-54). Both pro-apoptotic proteins Bcl-2 and Bax are transcriptionally controlled by p53 (55). The third peak of apoptosis takes place during the period of microvascularization, the final stage of lung development (2, 46, 50-51). The air-blood interface thins further; the double layer of capillaries is reduced to a single layer and the interstitial tissue decreases (48). All peaks of apoptosis during lung development are preceded by peaks in proliferation, indicating the importance of apoptosis for the removal of excess lung cells (46, 50-51). Thus, apoptosis is vital for proper lung development but the signaling cascades leading to cell death remain largely unknown.

Apoptosis and stretch

Premature born infants with respiratory insufficiency often require mechanical ventilation. Mechanical ventilation causes cellular stretch, which may trigger an inflammatory response and apoptosis (56-57). The mechanism by which mechanical stretch triggers apoptosis is unknown.

Stretch-induced apoptosis during lung development was first demonstrated in a fetal rabbits after tracheal occlusion (TO). Ligation of the trachea caused a depletion of alveolar type II cells due to increased apoptosis (49). A mechanical stretch simulating fetal breathing movements stimulated apoptosis of day 19 (canalicular stage) rat lung fibroblasts, but not epithelial type II cells, compared to control, unstretched cells (58). This observation and the finding of increased mesenchymal apoptosis during the canalicular stage *in vivo* (2, 42, 46) suggests a role for physiological stretch induced-apoptosis in lung remodelling during development.

The mechanisms by which excessive stretch of fetal lung cells contributes to the development of BPD are not well defined. A continuous cyclic stretch of 20% elongation for 24 hours, mimicking injurious ventilation, increased apoptosis and cell death of day 19 fetal rat type II cells and fibroblasts (58-59). Mechanical ventilation for 24 hours of newborn mice with air led to a 5-fold increase in the number of apoptotic lung cells (60). Unfortunately, the apoptotic lung cell type was not identified. *In utero* ventilation of fetal sheep for 6 hours with 21% oxygen increased the number of caspase-3 positive cells, which were mainly interstitial cells. (61). In both studies the ventilation-induced increase in apoptosis coincided with a significant reduction in alveolarization (60-61). So far only one study has investigated the effect of ventilation on apoptosis in infants with BPD. In lung sections from ventilated preterm infants delivered at 22-36 weeks the number of apoptotic epithelial cells increased significantly compared to stillborn fetuses (62). Besides ventilation, however, these infants were also treated with oxygen.

That apoptosis of fetal lung cells is triggered by stretch is now widely recognized, but the signalling pathways that mediate apoptosis remain to be elucidated. Stretch induces the activation of caspases (58, 60). It has been suggested that activation of the Fas/FasL system is pivotal in activating the caspases (43). FasL has a temporal pattern of expression in the developing lung (63-64) maximal during perinatal transition. The time-specific upregulation of FasL gene and protein expression during development suggests a tightly upregulated transcriptional (*or posttranscriptional*) regulation of the FasL gene. Fas is continuously expressed in the fetal rabbit lung (64), while in the murine lung its expression increases at birth (63-64). Both Fas and FasL immunolocalize to alveolar type II cells (63-64). The high FasL expression in distal epithelial cells during perinatal transition corresponds to increasing type II cell apoptosis and maximal airway distension (63-64) implicating Fas/FasL as a mediator of late-gestational apoptosis which may be mediated by stretch. Indeed, sustained lung expansion in fetal rabbits due to TO resulted in a time-specific increase of FasL in alveolar type II cells, which coincided with increased alveolar type II cell apoptosis (63). However, stretching of day 19 fetal rat fibroblasts induced apoptosis without detection of FasL (58). Hence, signalling pathways independent of FasL must be involved in stretch-induced apoptosis of fetal and neonatal lung fibroblasts.

Tumor necrosis factor (TNF)-α and its receptors represent another major extrinsic cell death pathway. Mechanical ventilation up-regulates TNF-α (65). Whether this pathway is important in stretch-induced apoptosis is unknown. Other pro-inflammatory cytokines/chemokines up-regulated by mechanical ventilation are interleukin (IL)-1β, IL-6, IL-8, and transforming growth factor (TGF)-β (59, 66-67). Some of these inflammatory mediators have also pro-apoptotic properties. The role of TGF-β in the pathogenesis of BPD has extensively been studied. Prolonged ventilation of newborn mice elevated pulmonary levels of Smad2, a downstream signaling molecule of the TGF-β pathway (68), indicating increased TGF-β signaling (60). TGF-β induces apoptosis by enhancing the Fas/FasL system via caspase-3 activation, but also via down regulation of $p21^{Cip1/Waf1}$ expression (43, 69). It has been shown that $p21^{Cip1/Waf1}$ suppresses apoptosis (70-71).

Apoptosis and hyperoxia

Oxygen is often life saving for prematurely born infants suffering from respiratory distress syndrome (RDS). To maintain adequate blood oxygen levels they require supraphysiological concentrations of oxygen. However, hyperoxia (or oxidative stress in a broader sense) is also a major contributing factor to the development of BPD (72-74). Oxidative stress is the result of an imbalance between exposure to oxidants and antioxidants. Prematurely born infants have a less developed and efficient antioxidant system (75-76) and oxygen treatment likely shifts the balance towards oxidative stress. In particular, free radicals and reactive oxygen species contribute to the development

of lung injury. These compounds have the ability to modify cellular macromolecules (proteins, lipids, carbohydrates and DNA), thereby promoting cell death (77-78).

Animal studies have shown that apoptosis is significantly increased in lungs exposed to hyperoxia. Exposure of newborn mice to 92% oxygen increased pro-apoptotic Bax mRNA and number of apoptotic lung cells (79). The increase in apoptosis was proportional to the duration of hyperoxia exposure. An increase in TUNEL-positive lung cells, mainly epithelial cells, and Bax expression was also demonstrated in neonatal rats exposed to > 90% oxygen (80). The increase in apoptosis coincided with an increase in p53, but not caspase-3, mRNA expression (81). Exposure of adult mice to 100% oxygen also increased p53 and Bax expression without affecting that of caspase-3 (82). Premature baboons delivered at 125 and 140 days of gestation and ventilated with high oxygen exhibited increased lung cell apoptosis and p53 expression (53, 83). Together, these findings suggest that p53 plays an essential role in hyperoxia-induced lung cell death. However, p53-deficient mice do not manifest an increased resistance to hyperoxia-induced lung damage (82), suggesting that multiple apoptotic pathways are involved in cell death during hyperoxia.

Interestingly, newborn wild-type mice raised in 95% oxygen had increased expression of pulmonary Fas and pro-apoptotic Bax, Bad and Bak mRNA, compared to newborn Fas-deficient *lpr* mice (84). Although apoptotic activity was similar in both groups, alveolar disruption was more severe in Fas-deficient *lpr* mice, suggesting a pro-proliferative rather than pro-apoptotic role of the Fas/FasL system (84). This contrasts to *in vitro* findings (85) and observations in adult *lpr* mice exposed to hyperoxia (82). These differences in pulmonary response to hyperoxia suggest that the effect of hyperoxia on pulmonary apoptosis and apoptosis-related gene expression may be age and species specific, which makes extrapolation of these data to the neonate complicated.

The TGF-β signaling pathway is also activated by hyperoxia. Neonatal mice and rats exposed to hyperoxia exhibited an arrest in alveolarization together with changes in the expression and localization of key signaling components of the TGF-β pathway (86-87). Exposure of alveolar type II cells to hyperoxia increased the sensitivity of these cells to TGFβ-induced apoptosis (86). Tracheal aspirates of extremely low birthweight neonates contain increased levels of bioactive TGF-β, which may be predictive of the need for oxygen therapy at home (67).

Besides activation of pro-apoptotic pathways, there is also evidence of decreased expression of anti-apoptotic pathways. Pulmonary pro-survival Akt expression was decreased in an experimental model of BPD induced by oxygen exposure of newborn rats (88). Akt over-expression preserved and restored normal alveolarization in this BPD model. This coincided with decreased protein expression of active caspase-3.

As mentioned earlier, clinically, mechanical ventilation is combined with oxygen treatment, making it hard to study the influence of each risk factor on cell death sepa-

rately. Lungs of ventilated and oxygen-treated preterm infants have increased apoptosis of alveolar epithelial cells (89). Caspase-3 appears activated but not caspase-8 and/or -9. The mechanism by which caspase-3 was activated in human neonates remains to be elucidated.

Apoptosis and inflammation

Inflammation plays a critical role in the pathogenesis of BPD (66, 90-92). Preterm infants developing BPD have increased numbers of inflammatory cells in their airways and elevated serum concentrations of interleukin (IL)-6, IL-8, IL-1β and IL-10 (93-95). An inflammatory response within the lungs of premature infants may result from prenatal (chorioamnionitis) and/or postnatal infections (bacteremia and sepsis), but can also be secondary to hyperoxia, hypoxia and mechanical ventilation or a combination of these factors. Each of these injurious stimuli triggers a different inflammatory response by activating different molecular pathways. In general, each stimulus increases the concentration of neutrophils, macrophages, proinflammatory cytokines and other inflammatory mediators.

Experimental animal models, mimicking chorioamnionitis in humans, have been used to study the effect of inflammation on lung development. In these models the effect of ventilation and oxygen is eliminated. Chorioamnionitis is an intra-uterine infection that starts early in pregnancy but remains undetectable until (preterm) delivery. It is associated with increased levels of pro-inflammatory cytokines in the amniotic fluid, including IL-6, IL-8, IL-1β, TNF-α and granulocyte colony stimulating factor (G-CSF) (91, 96-98). Infants of mothers with increased levels of pro-inflammatory cytokines in the amniotic fluid have a higher risk of developing BPD (91, 98). The incidence of chorioamnionitis in prematurely born infants varies between 33% and 71% (99). Antenatal infection induced by intra-amniotic injection of endotoxin to pregnant rats, rabbits or sheep results in a BPD-like disorder, characterized by pulmonary inflammation, reduced alveolar formation and vascularisation (100-103). Twenty four hours after being delivered via Caesarean section, lungs of premature lambs exposed to intra-amniotic endotoxin showed a transient increase in the number of apoptotic alveolar type II cells (104). Similar observations have been made in lung tissue of still born human fetuses exposed to chorioamnionitis. Exposure triggered an pulmonary inflammatory response and apoptosis of epithelial cells (89). The increase in apoptotic epithelial cells coincided with an increase in active caspase-8 (89). Fetuses that were exposed to chorioamnionitis and a pneumonia exhibited an even greater apoptotic index, suggesting a further increase of mediators inducing apoptosis or the release of other pro-apoptotic mediator such as fibronectin and elastase (89). BPD patients have increased concentrations of fibronectin in their tracheal lavage (105) and fibronectin has been implicated in regulating apoptosis during lung development (106). Elastase is

released by neutrophils and degrades elastin. Elastin is essential for alveolar development (107-108). In a newborn mouse model dysregulation of elastin was associated with increased lung cell apoptosis and impaired alveolar formation (107). TUNEL staining demonstrated significant more apoptotic cells, located in the walls of distal air spaces, and increased levels of active caspase-3. These mice were ventilated with 40% oxygen. The effect on apoptosis has also been demonstrated in a neonatal mouse model with a postnatal inflammation (109). Systemic lipopolysaccharide induced lung inflammation and a 2-fold increase in apoptotic alveolar epithelial cells in neonatal mice when compared to non-infectious controls (109).

Diminished apoptosis of inflammatory cells may also play a role in the development of BPD. It has been suggested that activated neutrophils in the lungs have an prolonged half-life due to delayed apoptosis by the macrophages (110). The delayed apoptotic response provides the neutrophils with a longer life span of 8 hours, resulting in accumulation of activated neutrophils at the local site of injury and inflammation. This may induce a persistent inflammatory state and delay repair processes. A potential mechanism for the diminished neutrophil apoptosis involves NFκB activation, which results in diminished caspase-3 and -9 activation (111-112) and decreased expression of pro-apoptotic proteins Bax, Bad and Bak (113). Inhibition of neutrophils influx

Table 1. Bronchopulmonary dysplasia and apoptotic molecules

Event	apoptotic molecules		Reference
	pro-apoptotic	anti-apoptotic	
Mechanical ventilation/	caspase-3		56, 58, 59
stretch	Fas/FasL		61
	TNF-α		63
	TGF-β		58, 65, 66
	IL-8, IL-6, IL-1β		57, 64
		IL-10	57
Hyperoxia	p53		53, 79, 81
	Bax		78, 82
	Bad, Bak	Fas/FasL	82
	TGF-β		84, 85
		Akt	86
Inflammation	caspase-3		107
	caspase-8		87
	IL-6, IL-8, IL-1β		
	TNF-α, G-CSF		89, 96-98
	Fas/FasL		
	fibronectine, elastase		87

in newborn rat lung with a selective CXC chemokine receptor-antagonist enhanced postnatal alveolar formation (114). Indeed, apoptosis of neutrophils in tracheal fluid of infants developing BPD is decreased (115-116). Impaired neutrophil apoptosis has been associated with a number of respiratory diseases (117), but its exact role in the pathogenesis of BPD is not known.

CONCLUSION

Inflammation, ventilation and oxygen are the most important risk factors for BPD and all three trigger apoptosis (Table 1). Abnormal apoptosis may contribute to the development of BPD. Apoptosis plays also an important role during normal growth and development of the lung as well as during repair after injury. Therefore, treatment strategies based on inhibiting apoptosis do carry the potential harm of inhibiting physiological apoptosis and inducing proliferation in cells other than targeted cells such as alveolar epithelial cells, fibroblasts and neutrophils. This can result in enhanced fibrosis and inefficient alveolar repair and recovery. A better understanding of the role of apoptosis in the pathogenesis of BPD and the signaling cascades leading to apoptosis is necessary before therapeutic strategies can be developed for the treatment of BPD.

REFERENCES

1. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972 Aug;26(4):239-57.
2. Scavo LM, Ertsey R, Chapin CJ, Allen L, Kitterman JA. Apoptosis in the development of rat and human fetal lungs. Am J Respir Cell Mol Biol. 1998 Jan;18(1):21-31.
3. Sherrill DL, Camilli A, Lebowitz MD. On the temporal relationships between lung function and somatic growth. Am Rev Respir Dis. 1989 Sep;140(3):638-44.
4. Polunovsky VA, Chen B, Henke C, Snover D, Wendt C, Ingbar DH, et al. Role of mesenchymal cell death in lung remodeling after injury. J Clin Invest. 1993 Jul;92(1):388-97.
5. Demedts IK, Demoor T, Bracke KR, Joos GF, Brusselle GG. Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema. Respir Res. 2006;7:53.
6. Kuwano K, Maeyama T, Inoshima I, Ninomiya K, Hagimoto N, Yoshimi M, et al. Increased circulating levels of soluble Fas ligand are correlated with disease activity in patients with fibrosing lung diseases. Respirology. 2002 Mar;7(1):15-21.
7. Hodge G, Nairn J, Holmes M, Reynolds PN, Hodge S. Increased intracellular T helper 1 proinflammatory cytokine production in peripheral blood, bronchoalveolar lavage and intraepithelial T cells of COPD subjects. Clin Exp Immunol. 2007 Oct;150(1):22-9.
8. Kasahara Y, Tuder RM, Cool CD, Voelkel NF. Expression of 15-lipoxygenase and evidence for apoptosis in the lungs from patients with COPD. Chest. 2000 May;117(5 Suppl 1): 260S.
9. Yokohori N, Aoshiba K, Nagai A. Increased levels of cell death and proliferation in alveolar wall cells in patients with pulmonary emphysema. Chest. 2004 Feb;125(2):626-32.
10. Albertine KH, Soulier MF, Wang Z, Ishizaka A, Hashimoto S, Zimmerman GA, et al. Fas and fas ligand are up-regulated in pulmonary edema fluid and lung tissue of patients with acute lung injury and the acute respiratory distress syndrome. Am J Pathol. 2002 Nov; 161(5):1783-96.
11. Matute-Bello G, Liles WC, Steinberg KP, Kiener PA, Mongovin S, Chi EY, et al. Soluble Fas ligand induces epithelial cell apoptosis in humans with acute lung injury (ARDS). J Immunol. 1999 Aug 15;163(4):2217-25.
12. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001 Jun; 163(7):1723-9.
13. Coalson JJ. Pathology of new bronchopulmonary dysplasia. Semin Neonatol. 2003 Feb; 8(1):73-81.
14. Jobe AJ. The new BPD: an arrest of lung development. Pediatr Res. 1999 Dec;46(6):641-3.
15. Bhandari A, Bhandari V. Pathogenesis, pathology and pathophysiology of pulmonary sequelae of bronchopulmonary dysplasia in premature infants. Front Biosci. 2003 May 1; 8:e370-80.
16. Lyon A. Chronic lung disease of prematurity. The role of intra-uterine infection. Eur J Pediatr. 2000 Nov;159(11):798-802.
17. Saugstad OD. Chronic lung disease: the role of oxidative stress. Biol Neonate. 1998 Sep; 74 Suppl 1:21-8.
18. Hennessy EM, Bracewell MA, Wood N, Wolke D, Costeloe K, Gibson A, et al. Respiratory health in pre-school and school age children following extremely preterm birth. Arch Dis Child. 2008 Dec;93(12):1037-43.

19. Baraldi E, Carraro S, Filippone M. Bronchopulmonary dysplasia: definitions and long-term respiratory outcome. Early Hum Dev. 2009 Oct;85(10 Suppl):S1-3.
20. Northway WH, Jr., Moss RB, Carlisle KB, Parker BR, Popp RL, Pitlick PT, et al. Late pulmonary sequelae of bronchopulmonary dysplasia. N Engl J Med. 1990 Dec 27;323(26): 1793-9.
21. Chipuk JE, Green DR. Do inducers of apoptosis trigger caspase-independent cell death? Nat Rev Mol Cell Biol. 2005 Mar;6(3):268-75.
22. Lionetti V, Recchia FA, Ranieri VM. Overview of ventilator-induced lung injury mechanisms. Curr Opin Crit Care. 2005 Feb;11(1):82-6.
23. Afford S, Randhawa S. Apoptosis. Mol Pathol. 2000 Apr;53(2):55-63.
24. Nicotera P, Leist M, Ferrando-May E. Apoptosis and necrosis: different execution of the same death. Biochem Soc Symp. 1999;66:69-73.
25. Tang PS, Mura M, Seth R, Liu M. Acute lung injury and cell death: how many ways can cells die? Am J Physiol Lung Cell Mol Physiol. 2008 Apr;294(4):L632-41.
26. Kroemer G, Martin SJ. Caspase-independent cell death. Nat Med. 2005 Jul;11(7):725-30.
27. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol. 1999;15:269-90.
28. Drakopanagiotakis F, Xifteri A, Polychronopoulos V, Bouros D. Apoptosis in lung injury and fibrosis. Eur Respir J. 2008 Dec;32(6):1631-8.
29. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004 Jan 23;116(2): 205-19.
30. Doseff AI. Apoptosis: the sculptor of development. Stem Cells Dev. 2004 Oct;13(5): 473-83.
31. Kavurma MM, Khachigian LM. Signaling and transcriptional control of Fas ligand gene expression. Cell Death Differ. 2003 Jan;10(1):36-44.
32. Kuwano K, Hagimoto N, Kawasaki M, Yatomi T, Nakamura N, Nagata S, et al. Essential roles of the Fas-Fas ligand pathway in the development of pulmonary fibrosis. J Clin Invest. 1999 Jul;104(1):13-9.
33. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science. 2004 Jul 30;305(5684):626-9.
34. Jaattela M. Multiple cell death pathways as regulators of tumour initiation and progression. Oncogene. 2004 Apr 12;23(16):2746-56.
35. Leist M, Jaattela M. Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol. 2001 Aug;2(8):589-98.
36. Tang PS, Tsang ME, Lodyga M, Bai XH, Miller A, Han B, et al. Lipopolysaccharide accelerates caspase-independent but cathepsin B-dependent death of human lung epithelial cells. J Cell Physiol. 2006 Nov;209(2):457-67.
37. Mantell LL, Lee PJ. Signal transduction pathways in hyperoxia-induced lung cell death. Mol Genet Metab. 2000 Sep-Oct;71(1-2):359-70.
38. Yasuhara S, Asai A, Sahani ND, Martyn JA. Mitochondria, endoplasmic reticulum, and alternative pathways of cell death in critical illness. Crit Care Med. 2007 Sep;35(9 Suppl): S488-95.
39. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. N Engl J Med. 2009 Oct 15; 361(16):1570-83.
40. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet. 2009;43:67-93.

41. Huang J, Klionsky DJ. Autophagy and human disease. Cell Cycle. 2007 Aug 1;6(15): 1837-49.
42. Burri PH. Lung development and pulmonary angiogenesis. In: Gaultier C, Bourbon JR, Post M, eds. Lung Development. New York: Oxford University Press, 1999:122-151.1999.
43. Del Riccio V, van Tuyl M, Post M. Apoptosis in lung development and neonatal lung injury. Pediatr Res. 2004 Feb;55(2):183-9.
44. Levesque BM, Vosatka RJ, Nielsen HC. Dihydrotestosterone stimulates branching morphogenesis, cell proliferation, and programmed cell death in mouse embryonic lung explants. Pediatr Res. 2000 Apr;47(4 Pt 1):481-91.
45. LJ. Wells EA, Boyden. In: The Developing Human Clinically Orientated Embryology. 6th ed. Philedelphia: WB Saunders, 1998:257-268 1998.
46. Kresch MJ, Christian C, Wu F, Hussain N. Ontogeny of apoptosis during lung development. Pediatr Res. 1998 Mar;43(3):426-31.
47. De Paepe ME, Gundavarapu S, Tantravahi U, Pepperell JR, Haley SA, Luks FI, et al. Fas-ligand-induced apoptosis of respiratory epithelial cells causes disruption of postcanalicular alveolar development. Am J Pathol. 2008 Jul;173(1):42-56.
48. Roth-Kleiner M, Post M. Similarities and dissimilarities of branching and septation during lung development. Pediatr Pulmonol. 2005 Aug;40(2):113-34.
49. De Paepe ME, Johnson BD, Papadakis K, Luks FI. Lung growth response after tracheal occlusion in fetal rabbits is gestational age-dependent. Am J Respir Cell Mol Biol. 1999 Jul; 21(1):65-76.
50. Bruce MC, Honaker CE, Cross RJ. Lung fibroblasts undergo apoptosis following alveolarization. Am J Respir Cell Mol Biol. 1999 Feb;20(2):228-36.
51. Schittny JC, Djonov V, Fine A, Burri PH. Programmed cell death contributes to postnatal lung development. Am J Respir Cell Mol Biol. 1998 Jun;18(6):786-93.
52. Haddad JJ. Oxygen homeostasis, thiol equilibrium and redox regulation of signalling transcription factors in the alveolar epithelium. Cell Signal. 2002 Oct;14(10):799-810.
53. Das KC, Ravi D. Altered expression of cyclins and cdks in premature infant baboon model of bronchopulmonary dysplasia. Antioxid Redox Signal. 2004 Feb;6(1):117-27.
54. Lakin ND, Jackson SP. Regulation of p53 in response to DNA damage. Oncogene. 1999 Dec 13;18(53):7644-55.
55. Tryka AF, Witschi H, Gosslee DG, McArthur AH, Clapp NK. Patterns of cell proliferation during recovery from oxygen injury. Species differences. Am Rev Respir Dis. 1986 Jun; 133(6):1055-9.
56. Uhlig S. Ventilation-induced lung injury and mechanotransduction: stretching it too far? Am J Physiol Lung Cell Mol Physiol. 2002 May;282(5):L892-6.
57. Hammerschmidt S, Kuhn H, Grasenack T, Gessner C, Wirtz H. Apoptosis and necrosis induced by cyclic mechanical stretching in alveolar type II cells. Am J Respir Cell Mol Biol. 2004 Mar;30(3):396-402.
58. Sanchez-Esteban J, Wang Y, Cicchiello LA, Rubin LP. Cyclic mechanical stretch inhibits cell proliferation and induces apoptosis in fetal rat lung fibroblasts. Am J Physiol Lung Cell Mol Physiol. 2002 Mar;282(3):L448-56.
59. Lee HS, Wang Y, Maciejewski BS, Esho K, Fulton C, Sharma S, et al. Interleukin-10 protects cultured fetal rat type II epithelial cells from injury induced by mechanical stretch. Am J Physiol Lung Cell Mol Physiol. 2008 Feb;294(2):L225-32.

60. Mokres LM, Parai K, Hilgendorff A, Ertsey R, Alvira CM, Rabinovitch M, et al. 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. Jan; 298(1):L23-35.
61. Allison BJ, Crossley KJ, Flecknoe SJ, Davis PG, Morley CJ, Hooper SB. Ventilation and oxygen: dose-related effects of oxygen on ventilation-induced lung injury. Pediatr Res. Mar;67(3):238-43.
62. May M, Strobel P, Preisshofen T, Seidenspinner S, Marx A, Speer CP. Apoptosis and proliferation in lungs of ventilated and oxygen-treated preterm infants. Eur Respir J. 2004 Jan; 23(1):113-21.
63. De Paepe ME, Mao Q, Luks FI. Expression of apoptosis-related genes after fetal tracheal occlusion in rabbits. J Pediatr Surg. 2004 Nov;39(11):1616-25.
64. De Paepe ME, Rubin LP, Jude C, Lesieur-Brooks AM, Mills DR, Luks FI. Fas ligand expression coincides with alveolar cell apoptosis in late-gestation fetal lung development. Am J Physiol Lung Cell Mol Physiol. 2000 Nov;279(5):L967-76.
65. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. Am J Respir Crit Care Med. 1999 Oct;160(4):1333-46.
66. Speer CP. Inflammation and bronchopulmonary dysplasia: a continuing story. Semin Fetal Neonatal Med. 2006 Oct;11(5):354-62.
67. Lecart C, Cayabyab R, Buckley S, Morrison J, Kwong KY, Warburton D, et al. Bioactive transforming growth factor-beta in the lungs of extremely low birthweight neonates predicts the need for home oxygen supplementation. Biol Neonate. 2000 May;77(4):217-23.
68. Moustakas A, Heldin CH. The regulation of TGFbeta signal transduction. Development. 2009 Nov;136(22):3699-714.
69. Hagimoto N, Kuwano K, Inoshima I, Yoshimi M, Nakamura N, Fujita M, et al. TGF-beta 1 as an enhancer of Fas-mediated apoptosis of lung epithelial cells. J Immunol. 2002 Jun 15; 168(12):6470-8.
70. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther. 2002 Jun;1(8):639-49.
71. Cazzalini O, Scovassi AI, Savio M, Stivala LA, Prosperi E. Multiple roles of the cell cycle inhibitor p21(CDKN1A) in the DNA damage response. Mutat Res. Apr-Jun;704(1-3):12-20.
72. Northway WH, Jr., Rosan RC. Radiographic features of pulmonary oxygen toxicity in the newborn: Bronchopulmonary dysplasia. Radiology. 1968 Jul;91(1):49-58.
73. Bancalari E, Claure N, Sosenko IR. Bronchopulmonary dysplasia: changes in pathogenesis, epidemiology and definition. Semin Neonatol. 2003 Feb;8(1):63-71.
74. Saugstad OD. Bronchopulmonary dysplasia-oxidative stress and antioxidants. Semin Neonatol. 2003 Feb;8(1):39-49.
75. Jain A, Mehta T, Auld PA, Rodrigues J, Ward RF, Schwartz MK, et al. Glutathione metabolism in newborns: evidence for glutathione deficiency in plasma, bronchoalveolar lavage fluid, and lymphocytes in prematures. Pediatr Pulmonol. 1995 Sep;20(3):160-6.
76. Russell GA. Antioxidants and neonatal lung disease. Eur J Pediatr. 1994;153(9 Suppl 2): S36-41.
77. Chabot F, Mitchell JA, Gutteridge JM, Evans TW. Reactive oxygen species in acute lung injury. Eur Respir J. 1998 Mar;11(3):745-57.
78. Morcillo EJ, Estrela J, Cortijo J. Oxidative stress and pulmonary inflammation: pharmacological intervention with antioxidants. Pharmacol Res. 1999 Nov;40(5):393-404.

79. McGrath-Morrow SA, Stahl J. Apoptosis in neonatal murine lung exposed to hyperoxia. Am J Respir Cell Mol Biol. 2001 Aug;25(2):150-5.
80. Husari AW, Dbaibo GS, Bitar H, Khayat A, Panjarian S, Nasser M, et al. Apoptosis and the activity of ceramide, Bax and Bcl-2 in the lungs of neonatal rats exposed to limited and prolonged hyperoxia. Respir Res. 2006;7:100.
81. Li YX, Luo XP, Liao LJ, Liu WJ, Ning Q. [Apoptosis in neonatal rat lung exposed to hyperoxia]. Zhonghua Er Ke Za Zhi. 2005 Aug;43(8):585-90.
82. Barazzone C, Horowitz S, Donati YR, Rodriguez I, Piguet PF. Oxygen toxicity in mouse lung: pathways to cell death. Am J Respir Cell Mol Biol. 1998 Oct;19(4):573-81.
83. Maniscalco WM, Watkins RH, Roper JM, Staversky R, O'Reilly MA. Hyperoxic ventilated premature baboons have increased p53, oxidant DNA damage and decreased VEGF expression. Pediatr Res. 2005 Sep;58(3):549-56.
84. Mao Q, Gundavarapu S, Patel C, Tsai A, Luks FI, De Paepe ME. The Fas system confers protection against alveolar disruption in hyperoxia-exposed newborn mice. Am J Respir Cell Mol Biol. 2008 Dec;39(6):717-29.
85. De Paepe ME, Mao Q, Chao Y, Powell JL, Rubin LP, Sharma S. Hyperoxia-induced apoptosis and Fas/FasL expression in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2005 Oct;289(4):L647-59.
86. Alejandre-Alcazar MA, Kwapiszewska G, Reiss I, Amarie OV, Marsh LM, Sevilla-Perez J, et al. Hyperoxia modulates TGF-beta/BMP signaling in a mouse model of bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol. 2007 Feb;292(2):L537-49.
87. Dasgupta C, Sakurai R, Wang Y, Guo P, Ambalavanan N, Torday JS, et al. Hyperoxia-induced neonatal rat lung injury involves activation of TGF-{beta} and Wnt signaling and is protected by rosiglitazone. Am J Physiol Lung Cell Mol Physiol. 2009 Jun;296(6):L1031-41.
88. Alphonse RS, Vadivel A, Coltan L, Eaton F, Barr AJ, Dyck JR, et al. Activation of Akt Protects Alveoli from Neonatal Oxygen-induced Lung Injury. Am J Respir Cell Mol Biol. Mar 26.
89. May M, Marx A, Seidenspinner S, Speer CP. Apoptosis and proliferation in lungs of human fetuses exposed to chorioamnionitis. Histopathology. 2004 Sep;45(3):283-90.
90. Kotecha S. Cytokines in chronic lung disease of prematurity. Eur J Pediatr. 1996 Aug;155 Suppl 2:S14-7.
91. Yoon BH, Romero R, Jun JK, Park KH, Park JD, Ghezzi F, et al. Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. Am J Obstet Gynecol. 1997 Oct;177(4):825-30.
92. von Bismarck P, Claass A, Schickor C, Krause MF, Rose-John S. Altered pulmonary interleukin-6 signaling in preterm infants developing bronchopulmonary dysplasia. Exp Lung Res. 2008 Dec;34(10):694-706.
93. Paananen R, Husa AK, Vuolteenaho R, Herva R, Kaukola T, Hallman M. Blood cytokines during the perinatal period in very preterm infants: relationship of inflammatory response and bronchopulmonary dysplasia. J Pediatr. 2009 Jan;154(1):39-43 e3.
94. Ambalavanan N, Carlo WA, D'Angio CT, McDonald SA, Das A, Schendel D, et al. Cytokines associated with bronchopulmonary dysplasia or death in extremely low birth weight infants. Pediatrics. 2009 Apr;123(4):1132-41.
95. Speer CP. Inflammation and bronchopulmonary dysplasia. Semin Neonatol. 2003 Feb;8(1):29-38.

96. Arcuri F, Toti P, Buchwalder L, Casciaro A, Cintorino M, Schatz F, et al. Mechanisms of leukocyte accumulation and activation in chorioamnionitis: interleukin 1 beta and tumor necrosis factor alpha enhance colony stimulating factor 2 expression in term decidua. Reprod Sci. 2009 May;16(5):453-61.
97. Holst RM, Laurini R, Jacobsson B, Samuelsson E, Savman K, Doverhag C, et al. Expression of cytokines and chemokines in cervical and amniotic fluid: relationship to histological chorioamnionitis. J Matern Fetal Neonatal Med. 2007 Dec;20(12):885-93.
98. Ghezzi F, Gomez R, Romero R, Yoon BH, Edwin SS, David C, et al. Elevated interleukin-8 concentrations in amniotic fluid of mothers whose neonates subsequently develop bronchopulmonary dysplasia. Eur J Obstet Gynecol Reprod Biol. 1998 May;78(1):5-10.
99. Been JV, Debeer A, van Iwaarden JF, Kloosterboer N, Passos VL, Naulaers G, et al. Early alterations of growth factor patterns in bronchoalveolar lavage fluid from preterm infants developing bronchopulmonary dysplasia. Pediatr Res. Jan;67(1):83-9.
100. Cao L, Wang J, Tseu I, Luo D, Post M. Maternal exposure to endotoxin delays alveolarization during postnatal rat lung development. Am J Physiol Lung Cell Mol Physiol. 2009 May;296(5):L726-37.
101. Gras-Le Guen C, Denis C, Franco-Montoya ML, Jarry A, Delacourt C, Potel G, et al. Antenatal infection in the rabbit impairs post-natal growth and lung alveolarisation. Eur Respir J. 2008 Dec;32(6):1520-8.
102. Jobe AH, Newnham JP, Willet KE, Sly P, Ervin MG, Bachurski C, et al. Effects of antenatal endotoxin and glucocorticoids on the lungs of preterm lambs. Am J Obstet Gynecol. 2000 Feb;182(2):401-8.
103. Kallapur SG, Bachurski CJ, Le Cras TD, Joshi SN, Ikegami M, Jobe AH. Vascular changes after intra-amniotic endotoxin in preterm lamb lungs. Am J Physiol Lung Cell Mol Physiol. 2004 Dec;287(6):L1178-85.
104. Kramer BW, Kramer S, Ikegami M, Jobe AH. Injury, inflammation, and remodeling in fetal sheep lung after intra-amniotic endotoxin. Am J Physiol Lung Cell Mol Physiol. 2002 Aug; 283(2):L452-9.
105. Gerdes JS, Yoder MC, Douglas SD, Paul M, Harris MC, Polin RA. Tracheal lavage and plasma fibronectin: relationship to respiratory distress syndrome and development of bronchopulmonary dysplasia. J Pediatr. 1986 Apr;108(4):601-6.
106. Roman J. Fibronectin and fibronectin receptors in lung development. Exp Lung Res. 1997 Mar-Apr;23(2):147-59.
107. Bland RD, Ertsey R, Mokres LM, Xu L, Jacobson BE, Jiang S, et al. Mechanical ventilation uncouples synthesis and assembly of elastin and increases apoptosis in lungs of newborn mice. Prelude to defective alveolar septation during lung development? Am J Physiol Lung Cell Mol Physiol. 2008 Jan;294(1):L3-14.
108. Bland RD, Xu L, Ertsey R, Rabinovitch M, Albertine KH, Wynn KA, et al. Dysregulation of pulmonary elastin synthesis and assembly in preterm lambs with chronic lung disease. Am J Physiol Lung Cell Mol Physiol. 2007 Jun;292(6):L1370-84.
109. Alvira CM, Abate A, Yang G, Dennery PA, Rabinovitch M. Nuclear factor-kappaB activation in neonatal mouse lung protects against lipopolysaccharide-induced inflammation. Am J Respir Crit Care Med. 2007 Apr 15;175(8):805-15.
110. Chopra M, Reuben JS, Sharma AC. Acute lung injury:apoptosis and signaling mechanisms. Exp Biol Med (Maywood). 2009 Apr;234(4):361-71.

111. Taneja R, Parodo J, Jia SH, Kapus A, Rotstein OD, Marshall JC. Delayed neutrophil apoptosis in sepsis is associated with maintenance of mitochondrial transmembrane potential and reduced caspase-9 activity. Crit Care Med. 2004 Jul;32(7):1460-9.
112. Cheah FC, Hampton MB, Darlow BA, Winterbourn CC, Vissers MC. Detection of apoptosis by caspase-3 activation in tracheal aspirate neutrophils from premature infants: relationship with NF-kappaB activation. J Leukoc Biol. 2005 Mar;77(3):432-7.
113. Hanna N, Vasquez P, Pham P, Heck DE, Laskin JD, Laskin DL, et al. Mechanisms underlying reduced apoptosis in neonatal neutrophils. Pediatr Res. 2005 Jan;57(1):56-62.
114. Yi M, Jankov RP, Belcastro R, Humes D, Copland I, Shek S, et al. Opposing effects of 60% oxygen and neutrophil influx on alveologenesis in the neonatal rat. Am J Respir Crit Care Med. 2004 Dec 1;170(11):1188-96.
115. Oei J, Lui K, Wang H, Henry R. Decreased neutrophil apoptosis in tracheal fluids of preterm infants at risk of chronic lung disease. Arch Dis Child Fetal Neonatal Ed. 2003 May; 88(3):F245-9.
116. Kotecha S, Mildner RJ, Prince LR, Vyas JR, Currie AE, Lawson RA, et al. The role of neutrophil apoptosis in the resolution of acute lung injury in newborn infants. Thorax. 2003 Nov; 58(11):961-7.
117. Bianchi SM, Dockrell DH, Renshaw SA, Sabroe I, Whyte MK. Granulocyte apoptosis in the pathogenesis and resolution of lung disease. Clin Sci (Lond). 2006 Mar;110(3):293-304.

Chapter 5

Mechanical Ventilation-Induced Apoptosis in Newborn Rat Lung is mediated via FasL/Fas Pathway

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Submitted

ABSTRACT

Rationale: Mechanical ventilation induces pulmonary apoptosis and inhibits alveolar development in preterm infants, but the molecular basis for this apoptotic injury is unknown. *Objective*: To determine the signaling mechanism(s) of ventilation(stretch)-induced apoptosis in newborn rat lung. *Methods:* Seven-day old rats were ventilated with room air for 24 h using moderate tidal volumes (8.5 $mL.kg^{-1}$). Isolated fetal rat lung epithelial and fibroblast cells were subjected to continuous cyclic stretch (5, 10 or 17% elongation) for up to 12 h. *Measurements and Main Results:* Prolonged ventilation increased significantly the number of apoptotic alveolar type II cells (i.e. TUNEL-labelling, anti-cleaved caspase-3 immunochemistry) and was associated with increased expression of the apoptotic mediator FasL. Fetal lung epithelial cells, but not fibroblasts, subjected to maximal (i.e. 17%, but not lesser elongation) cyclic stretch exhibited increased apoptosis (i.e. nuclear fragmentation; DNA laddering) which appeared to be mediated *via* the extrinsic pathway (increased expression of FasL and cleaved caspase-3, -7 and -8). The intrinsic pathway appeared not to be involved (minimal mitochondrial membrane depolarization (i.e. JC-1 flow analysis); no activation of caspase-9). Universal caspases inhibition and neutralization of FasL abrogated the stretch-induced apoptosis. *Conclusion:* Prolonged mechanical ventilation induces apoptosis of alveolar type II cells in newborn rats and the mechanism appears to involve activation of the extrinsic death pathway *via* the FasL/Fas system.

INTRODUCTION

Mechanical ventilation is frequently required in preterm infants. Although contemporary 'protective' ventilation is designed to minimize volutrauma (1-2), this has not reduced the incidence of bronchopulmonary dysplasia (BPD) (3), a disease characterized by arrest of alveolar development. How mechanical ventilation adversely affects alveolar development is unknown, but a potentially important mechanism is apoptosis. For example, apoptotic cell (particularly alveolar epithelial cells) numbers are increased in ventilated pre-term infants (4-6). Apoptosis is central to -and occurs in all stages of- lung development (6-9), and abnormal apoptotic activity may reduce alveolar number. Indeed, prolonged mechanical ventilation of neonatal mice increased lung epithelial cell apoptosis which was paralleled by inhibition of alveolar development (10).

Apoptosis can occur through the extrinsic or intrinsic (mitochondrial) pathways, but the pathway related to ventilator-induced apoptosis in the preterm lung is not known. Emerging evidence suggests a role for the extrinsic (Fas Ligand (FasL)/Fas) system in lung development (11). Fas is continuously expressed in fetal rabbit lung (12), in murine lung its expression increases at birth (9), and both Fas and FasL immunolocalize to bronchial epithelial cells and alveolar type II cells (9, 12). The temporal expression of FasL throughout development suggests transcriptional (or posttranscriptional) regulation of the FasL gene, and its expression in distal epithelial cells during perinatal transition corresponds to maximal airway distension and increasing apoptosis (i.e. in type II epithelial cells) (9, 12); thus, FasL-Fas may mediate stretch-induced apoptosis of late gestation. Indeed, this is consistent with the finding that tracheal occlusion (a potent cause of lung stretch) increases type II cell apoptosis as well as FasL protein in the fetal lung (13). Finally, induction of lung-specific FasL (using inducible transgenic mice) causes increased lung epithelial apoptosis during postcanalicular remodeling and impaired alveolar development (14). However, the role of the Fas/FasL system in ventilation-induced apoptosis in preterm infants remains is unknown. Mechanical ventilation (stretch) may also activate the mitochondrial apoptotic pathway, leading to activation of initiator caspase-9. This pathway has received little attention in preterm infants. Such infants, when ventilated, have increased levels of cleaved-caspase 3, but not caspases-8 or -9 (5). Thus, it is unclear by which pathway apoptosis is activated during mechanical ventilation of immature lungs.

In this study we demonstrated *in vivo* (mechanical ventilation) and *in vitro* (cell stretch) stretch-induced apoptosis in pre-term and newborn lung models, and that apoptosis develops in type II epithelial cells (but not fibroblasts), and that the mechanism is through activation of the extrinsic (not intrinsic) pathway.

METHODS

Animals*:* Female timed-pregnant Wistar rats were obtained from Charles River (St. Quebec, Canada) and were housed in the Hospital for Sick Children animal facilities until used. All animal procedures were in accordance with Canadian Council of Animal Care guidelines and were approved by the Animal Care Reveiw Committee of the Hospital for Sick Children.

Mechanical ventilation: Seven-day old rat pups were anesthetized by *i.p*. injection of pentobarbital (30 $mg.kg^{-1}$) and a tracheotomy was performed. Isoflurane was used as general anesthesia during the ventilation period and 0.9% saline (100 $ml.kg^{-1}$) was administered subcutaneously to prevent dehydration. Rat pups were ventilated (FlexiVent Scireq, Montreal, PQ) with room air and moderate V_T (7-9 $ml.kg^{-1}$, RR 150 min^{-1}, PEEP 2 cm H_2O) for 24 h as previously described (15) Rectal temperature was maintained at 37°C using a thermal blanket, lamp and plastic wrap. At the end of ventilation lung tissues were processed for histology or fresh frozen for molecular/protein analyses.

Immunohistochemistry: After flushing whole lungs were infused in situ with 4% (wt/vol) paraformaldehyde (PFA) in PBS with a constant pressure of 20 cm H_2O over 5 minutes to have equalized filling pressure over the entire lung. Under these constant pressure conditions the cannula was removed and the trachea immediately ligated. The lungs were excised and immersed in 4% PFA in PBS overnight, dehydrated in a ethanol/xylene series and embedded in paraffin. Following sectioning and antigen retrieval by heating in 10 mM sodium citrate pH 6.0, sections were washed in PBS and endogenous peroxidase was blocked in 3% (v/v) H_2O_2 in methanol. Blocking was done with 5% (w/v) normal goat serum (NGS) and 1% (w/v) bovine serum albumin (BSA) in PBS. Sections were then incubated overnight at 4°C with either rabbit anti-cleaved caspase 3 (Asp 175) antibody (1:200 dilution; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Egr1 (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-FasL (1:500 dilution; Thermo Scientific Inc., Ottawa, ON, CA). Biotinylated goat anti-rabbit IgG was used as secondary antibody at a 1:300 dilution. Color detection was performed according to instruction in the Vectastain ABC and DAB kit (Vector Laboratories, Burlingname, CA, USA). Sections were counterstained with hematoxylin.

Immunofluorescence double staining: Following blocking sections were first incubated with rabbit anti-FasL (1:500 dilution; Thermo Scientific Inc., Ottawa, ON, CA) and then with biotinylated goat anti-rabbit IgG (dilution 1:300) and fluorescein-streptavidin (Invitrogen Corporation, Carlsbad, California, USA). Subsequently, sections were blocked again and then incubated with rabbit anti-prosurfactant protein C (pro-SPC)(1:1000

dilution, Abcam, Cambridge, USA), biotinylated goat anti-rabbit IgG (dilution 1:300) and CY™3-streptavidin (Invitrogen).

TUNEL Assay: Sections were treated with proteinase K, washed in PBS and the terminal transferase dUTP end-labeling (TUNEL) assay was conducted according to manufacturer's instructions (in situ cell death detection-POD Kit; Roche, Montreal, Quebec, Canada). Following incubation with anti-fluorescein antibody staining was visualised using DAB. Sections were counterstained with hematoxylin. For quantitative analysis, digital images were captured at 20× magnification with random sampling of all tissue in an unbiased fashion. Images were captured randomly from 15 non-overlapping fields/slide with 3 slides/animal and 4 animals/group.

Cell stretch: Timed pregnant rats and their fetuses were sacrificed at day 19 of gestation (term=22 days). Rat fetal lung fibroblast (FLF) and epithelial (FLDE) cells were isolated as described previously (16). Within 24 hours of isolation, fibroblast and epithelial cells were separately inoculated at a density of 10^6 cells/well on Bioflex multiwell plates precoated with collagen I and maintained in MEM + 10% FBS. Six hours prior to stretch medium was changed to MEM + 0.5% FBS. Cells received fresh MEM + 0.5% FBS and were then mounted in a Flexercell FX-4000 Strain Unit (Flexercell Int., Hillsborough, NC). Cyclic continuous radial elongations of 5, 10 or 20% were applied at intervals of 30 cycles per min for 2, 4, 6 and 12 h. In some cases, cyclic intermittent radial elongation of 5% at an interval of 30 cycles per min, 15 min/hour, was applied. Control cells were grown on the Bioflex collagen I plates, treated in the same manner as stretched cells, but were subjected to no stretch. Cells treated for 3 h with staurosporine, which induced apoptosis, served as positive control.

Analysis of DNA fragmentation: Following exposure to stretch, cells were stained with DAPI to visualize nuclear fragmentation. Apoptosis was confirmed by analysis of oligonucleosomal DNA cleavage. For DNA extraction, cells were incubated overnight at 55°C in lysis buffer (10mM Tris HCl, pH7.5, 10mM EDTA, 0.1% SDS, 0.2% Triton X-100, and 0.1 mg/ml proteinase K). After overnight lysis at 50°C, the samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was then precipitated with ethanol and sodium acetate and resuspended in Tris-EDTA, pH 7.5. The ApoAlert LM-PCR Ladder Assay Kit (Clontech Laboratories, Palo Alto, CA) was used for the detection of nucleosomal ladders in apoptotic cells, according to the manufacturer's instructions. Amplified products were visualized by electrophoresis in 1.5% agarose ethidium-bromide gels.

Analysis of PARP activity: After stretching, cells were trypsinized and resuspended in MEM (1.5 x 10^5 cells/ ml). The cell suspension was centrifuged at 1,200x*g* for 3 min and the supernatant was discarded. The pellet was resuspended in 1 ml of 56 mM Hepes, 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$ and vortexed. A 500 µl aliquot of cell suspension was added to pre-mixed Eppendorf tubes containing 25 µl digitonin, 25 µl Nicotinamide Adenine Dinucleotide (NAD) and 3 µl [^{3}H]-NAD (ICN Biomedicals, St. Laurent, PQ) and incubated at 37°C for 5 minutes. After the incubation 200 µl of ice-cold 50% (vol/vol) TCA was added to the tube and left on ice for 10 minutes. The tubes were then centrifuged at 10,000x*g* for 10 min. The supernatant was discarded and the pellet was washed twice with ice-cold 20% (vol/vol) TCA. The final pellet was solubilized by the addition of 100 µl of 4% (vol/vol) SDS and 100 µl of 0.2M NaOH. The tubes were incubated overnight at 37°C. The next day the tubes were gently vortexed and 200 µl of the solubilized samples were placed in scintillation tubes. Four ml of cytoscint (Ready-Safe, Millipore, Billerica, MA) and 30 µl 100% acetic acid were also added to the scintillation tubes. The tubes were gently vortexed to ensure that everything was mixed and liquid scintillation counting was performed. PARP activity in counts per minute (cpm/10^6 cells), was expressed as fold change of PARP activity versus control samples.

Analysis of membrane permeability: After stretching, cells were harvested and washed twice with cold PBS. The cell density was adjusted to 1x10^6 cells per ml in PBS. One µl of 100 µM YO-PRO-1 and 1.5 mM Propidium Iodine (Molecular Probes, Eugene, OR) were added to one ml of cell suspension. The cells were then incubated on ice for 30 minutes in the dark and analyzed by flow cytometry (FACS Calibur using CELLQuest software; Becton Dickinson, Mountain View, CA), measuring the fluorescence emission at 530 nm and >575 nm.

Analysis of mitochondrial membrane potential: After stretching, cells were harvested, adjusted to a density of 0.5x10^6 per ml and stained with 2 µg/ml JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide: Molecular Probes, Eugene, OR) dye for 30 min at 37 °C. Cells were then washed twice with cold PBS containing 1% BSA and 7 mM sodium azide. Cells were resuspended in PBS and analyzed by flow cytometry. Mitochondrial membrane depolarization was monitored as a result of a shift in emission spectra after 480 nm excitation from orange-red (590 nm, for JC-1 aggregates) to green (530 nm, for JC-1 monomers). The green and red fluorescence signals were resolved by detection in the conventional FL1 and FL2 channels, respectively.

Caspase activity measurements: Activation of caspases was determined by fluorometry. After stretch, cells were harvested and protein lysates were extracted. Lysates were

incubated with and without either caspase 3 (DEVD-fmk), caspase 8 (IETD-fmk) or caspase 9 (LEHD-fmk) substrates (Biomol, Plymouth Meeting, PA) for 2 hours at 37°C in 96-well bottom black coated plates (Sigma, St. Louis, MO). Activity was measured on microplate reader set a wavelength of 400 nm. The relative increase in caspase activity in stretched cells versus static control cells was calculated after subtracting the background obtained from lysates containing no substrate.

Western blot Analysis: Total protein was extracted from cells and protein content was measured according to Bradford (17). Samples (40 g protein) were subjected to 10% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% skim milk in TBST (20mM Tris, 137 mM NaCl, 0.1% Tween 20) membranes were incubated with appropriate primary antibody overnight in 4°C. Primary antibodies were rabbit anti-cleaved caspase 8, (dilution of 1:500), rabbit anti-cleaved caspase 7 (dilution of 1:1000), rabbit anti-cleaved caspase 3 (dilution 1:500), rabbit anti-cleaved caspase 9 (dilution 1:500) and rabbit anti-FasL (dilution of 1:1000). The next day the membranes were washed TBST and incubated with either horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG (dilution of 1:1000; Cell Signaling Technology, Danvers, USA). After several washes with TBST, protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ, USA). Equal protein loading was confirmed by β-actin immunoblotting of same membrane.

Inhibition of Caspase Activation: Two broad spectrum caspase inhibitors benzylocarbonyl-Val-Ala-Asp-fluromethylketone (z-VAD-fmk) (R&D, Minneapolis, MN) and IDN6556 ((Idun Pharmaceuticals; donated by Dr. Mingyao Liu (University Hospital Network, Toronto, ON)) were dissolved in DMSO. Cells were pretreated with either medium containing DMSO or inhibitor (1-10 μM) for the duration of the stretch regiment. After the stretch, the cells were analyzed for DNA-laddering and caspase-3 activity.

Neutralizing FasL: Cells were stretched in the presence and absence of 3μg/ml of either purified hamster anti-(mouse and rat) CD178 (Fas Ligand) antibody or nonimmune hamster IgG (BD Biosciences, Pharmingen, San Jose, CA) followed by assessment of cl-caspase-3 and -8 expression, caspase 8 activation and DNA fragmentation.

Quantitative RT-PCR: Total RNA was extracted from either lung tissues or cells and reverse transcribed. Complementary DNA was amplified for target genes Egr1, Fas and FasL as previously described (18-19). For relative quantification, polymerase chain reaction signals were compared between groups after normalization using 18S as internal reference. Fold change was calculated (20).

Statistical Analysis: All values of animal experiments are presented as mean ± SEM of at least 4 separate animals. All values of cell experiments are shown as means ± SD of at least 3 separate experiments carried out in triplicate. Statistical analysis was by Student's *t* test or, for comparison of more than two groups, by one-way analysis of variance followed by Duncan's multiple range comparison test, with significance defined as $p < 0.05$.

RESULTS

In Vivo Stretch-induced Apoptosis

Prolonged (24 h) mechanical ventilation with moderate V_T significantly increased the number of TUNEL-positive cells in the lungs of 7-day old rat pups *vs*. non-ventilated 8-day rat pups (Fig. 1). The increase in lung cell apoptosis by prolonged ventilation was confirmed by anti-cleaved caspase-3 immunohistochemistry (Fig. 1A:d-f). In adult rat lung, high V_T rapidly induces early growth response (Egr)-1, a transcription factor implicated in regulating FasL expression (21-22). Therefore, we investigated the effect of prolonged ventilation with moderate V_T on Egr-1 and FasL expression in newborn rat lung. Both Egr-1 and FasL mRNA and protein levels were increased by 24 h ventilation (Fig. 2A), and immunohistochemical localization indicated up-regulation primarily in alveolar epithelial type II cells (Fig. 2B), in agreement with previous findings for Egr-1 in adult lung (23). Co-localization of FasL and pro-SPC (type II cell marker) immunofluorescence confirmed the presence of FasL in type II epithelial cells (Fig. 2C).

We then utilized rat fetal lung distal epithelial (FLDE) cells in order to investigate the mechanism of ventilation (stretch)-induced apoptosis.

In Vitro Stretch-induced Apoptosis

Nuclear fragmentation and DNA laddering were detected in FLDE cells subjected to continuous 17% cyclic stretch (mimics moderate/high V_T ventilation) of 6 h when compared to static cultured control cells (Fig. 3A). This cell stretch regimen also increased PARP activity in FLDE cells but did not trigger DNA fragmentation or changes in PARP activity in fetal lung fibroblast (FLF) cells (Fig. 3B). Additionally, FLDE membrane permeability (i.e. fluorescent dye YO-PRO-1 absorbance) was increased compared to control cells (Fig. 3C). After 6 hours of stretch, membrane transport became transiently defective and the rate of uptake of the YO-PRO-1 was significantly increased (1.5×fold increase *vs*. static control; $p < 0.001$), and was further increased (1.7×fold *vs*. control; $p < 0.001$) after 12 hours of stretch. This stretch-induced permeability increase over time correlated with increased DNA fragmentation (Fig. 4A). Together, these data suggest

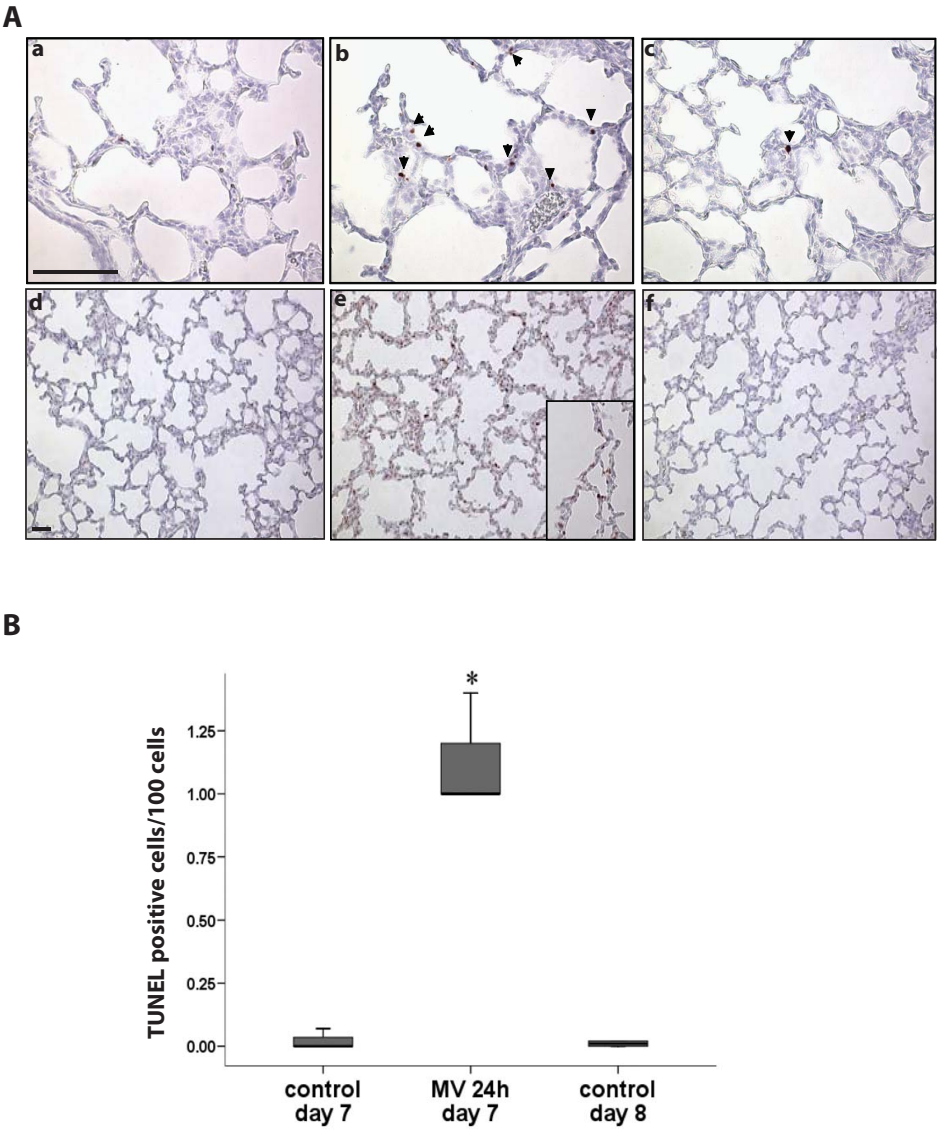

Figure 1. Prolonged ventilation of newborn rat with moderate V_T triggers pulmonary apoptosis. TUNEL analysis (**A, a-c**) and cleaved caspase-3 immunohistochemistry (**A, d-f**) revealed increased numbers of apoptotic (brownish) cells (arrows) in 7-day newborn rat lung ventilated for 24 h with V_T of 7-9 $ml.kg^{-1}$ (**A-b, A-e**) when compared to non-ventilated 7- (**A-1, A-d**) and 8-day (**A-c, A-f**) newborn rat lungs. (**B**) Quantification of number of TUNEL-positive cells in ventilated and non-ventilated control rat lungs.

that a continuous 17% cyclic stretch regimen rapidly induces apoptosis in FLDE cells. To determine if lower levels of stretch would also initiate an apoptotic response in FLDE cells, cells were subjected to lesser stretch regimens. Neither intermittent stretch (5%; mimics fetal breathing movements) or continuous cyclic stretch (mimics regular

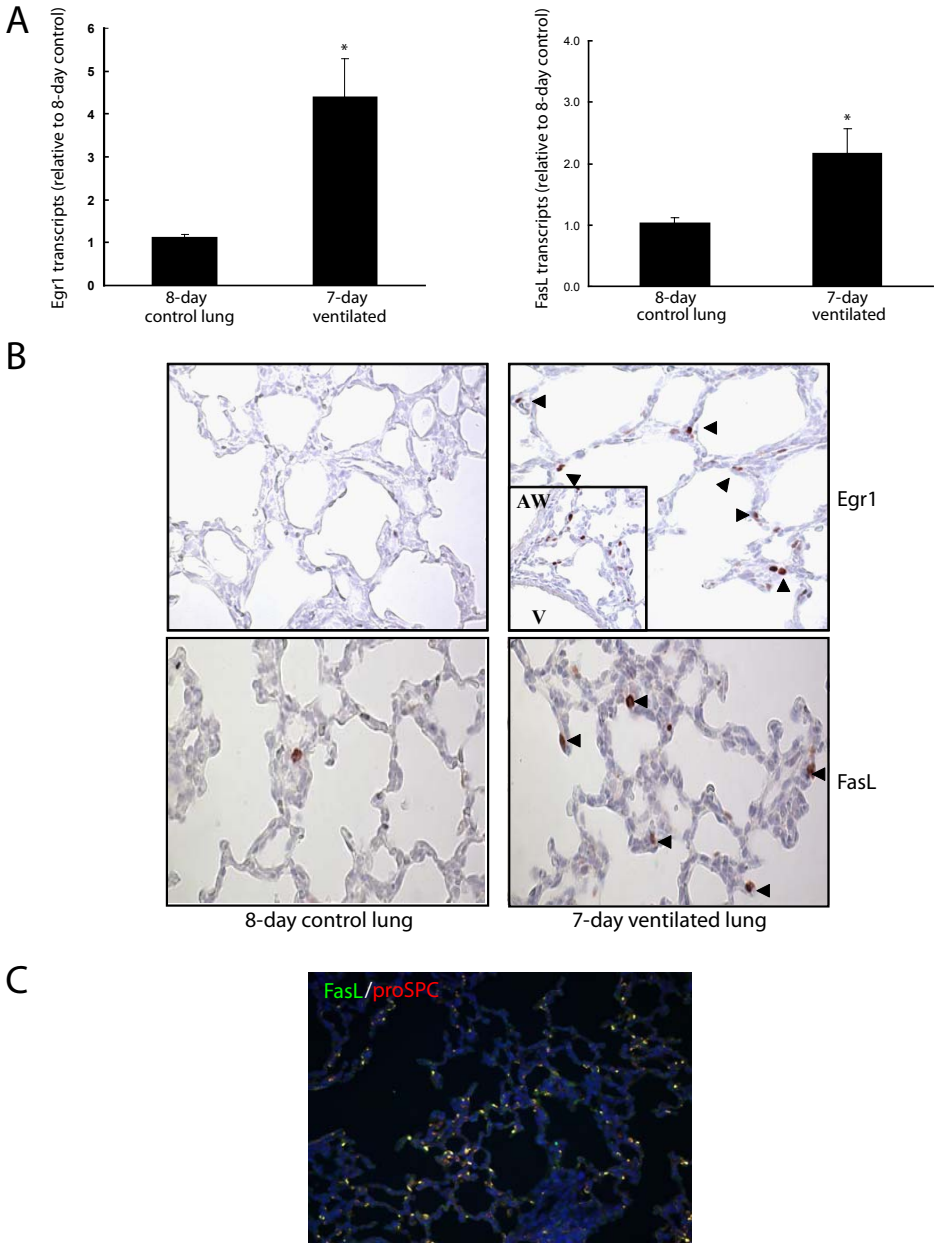

Figure 2. Prolonged ventilation of newborn rat with moderate V_T induces pulmonary Egr1 and FasL expression. A 24-h ventilation of 7-day rat with V_T of 7-9 ml.kg^{-1} increased number of Egr1 and FasL transcripts (**A**) and immunopositive (brownish) cells (**B**) in whole lung compared to 8-day non-ventilated rat lung. (**C**) FasL (green) immunolocalizes with pro-SPC (alveolar type II cell marker: red) in the ventilated newborn rat lung (merged yellow indicates co-localization).

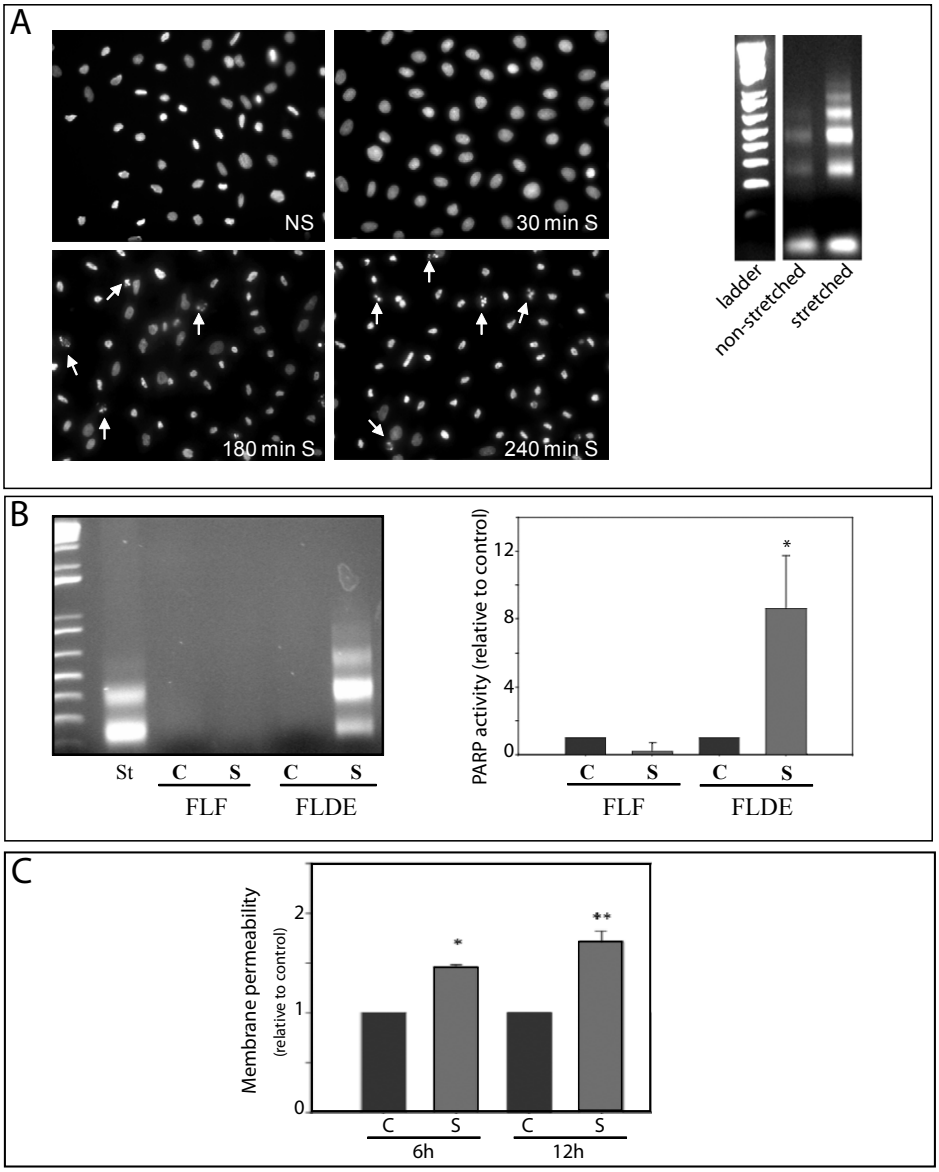

Figure 3. A continuous 17% cyclic stretch induces apoptosis in fetal lung epithelial cells. Increases in nuclear fragmentation (**A, left panel** - arrows), DNA laddering (**A-right panel** and **B-left panel**), PARP activity (**B-right panel**) and membrane permeability (**C**) were noted fetal lung distal epithelial (FLDE) cells, but not fetal lung fibroblasts (**B**), after 6 (**A, B, C**) and 12 (**C**) hours of stretch. C = static control; S = stretch; FLF: fetal lung fibroblasts. * or **$p < 0.05$ vs. 6 or 12 h controls

breathing) did not induce DNA fragmentation, nor did the membrane permeability change in FLDE cells (Figure 4-B,C). Longer duration of stretch (17% cyclic stretch for 72 hours) also induced apoptosis in fetal lung fibroblasts (Fig. 4D).

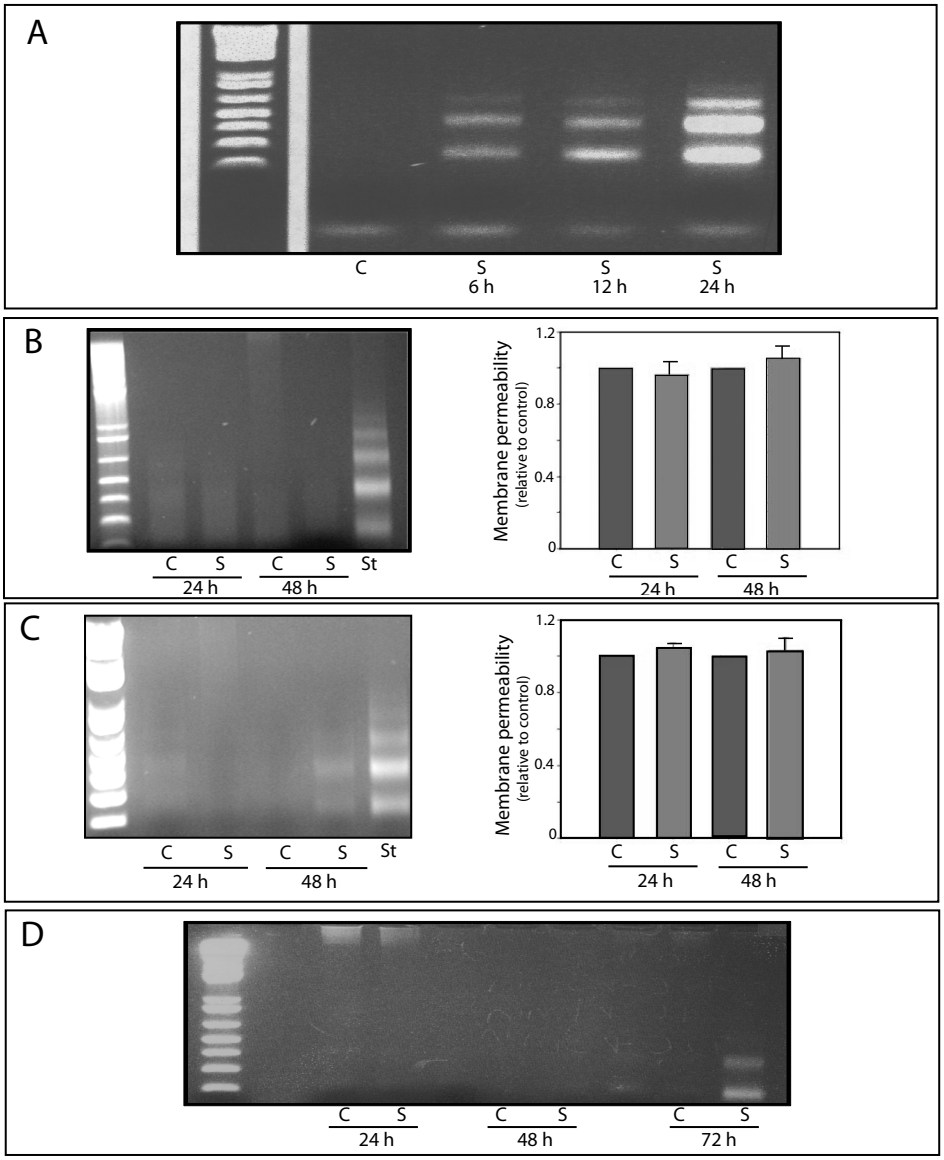

Figure 4. Stretch-induced apoptosis is time-, amplitude- and cell type-dependent. **(A)** Increased DNA fragmentation in rat fetal lung epithelial cells with duration of continuous 17% cyclic stretch. (**B and C**) Exposure of rat fetal lung epithelial cells to either an intermittent 5% cyclic stretch (**B**) or continuous 10% cyclic stretch (**C**) for 24-48 hours did not trigger DNA fragmentation (left panels) or alter the membrane permeability of the cells (right panels). (**D**) DNA fragmentation occurred in rat fetal lung fibroblast after 72 hours of continuous 17% cyclic stretch. C = static control; S = stretch.

Stretch-induced Apoptosis – Role of Caspases: Universal inhibitors of caspases (*ZVAD-fmk and IDN6556*) abrogated the stretch-induced DNA laddering in FLDE cells (Fig. 5A). Indeed, stretch increased the amount of cleaved-caspase-3, -7 and -8 (Fig. 5B)

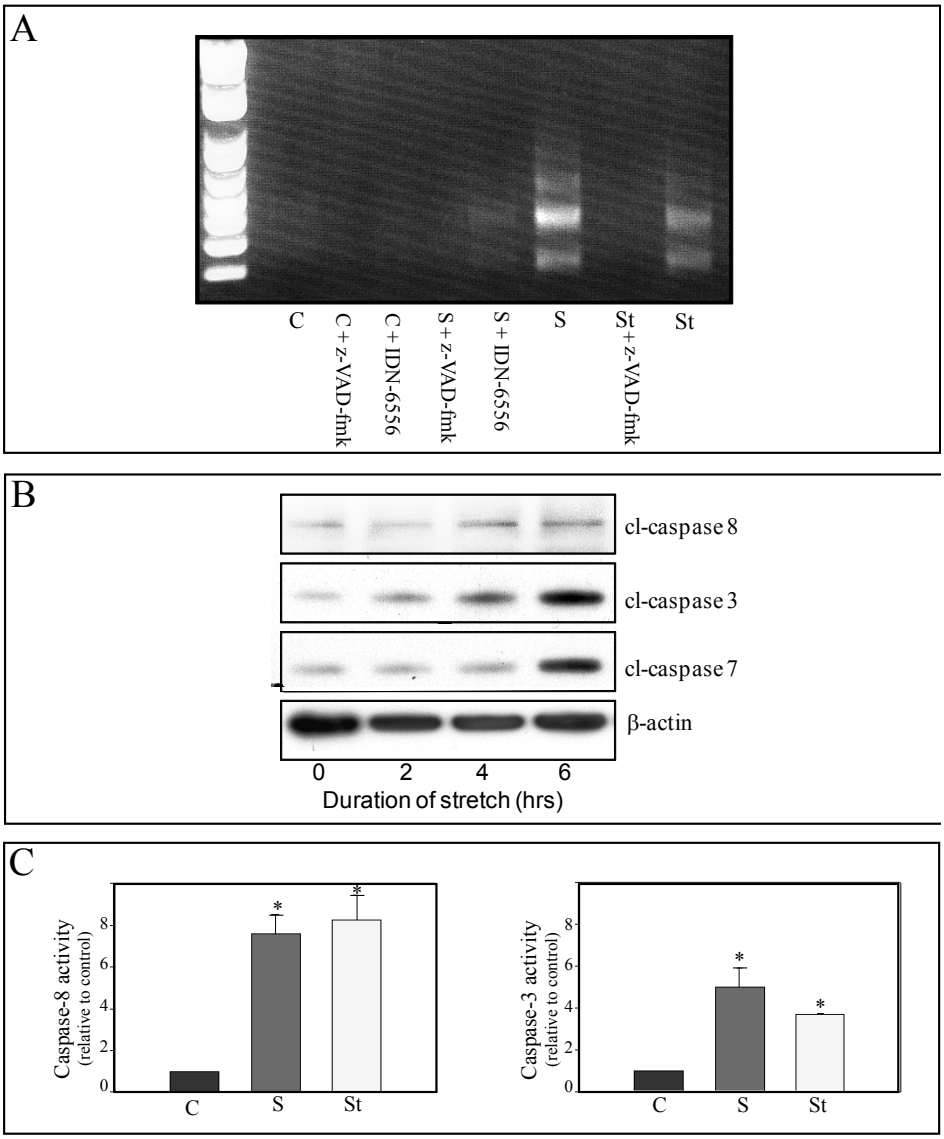

Figure 5. Stretch-induced apoptosis in fetal lung epithelial cells is caspase-dependent. (**A**) Universal caspase inhibitors, z-VAD-fmk and IDN-6556, abrogate DNA fragmentation in fetal lung epithelial cells subjected to a continuous 17% cyclic stretch of 6 hours. (**B**) Cleaved caspase-3, -7 and -8 content increases with duration of 17% stretch. (**C**) Increases of caspase-3 and -8 activities after 6 hours of 17% stretch. C = static control; S = stretch; St = staurosporine positive control; *$p < 0.05$.

as well as the activities of caspase-3 and -8 (Fig. 5C), implying activation of the extrinsic death pathway. Activation of the mitochondrial (intrinsic) death pathway was determined by measuring caspase-9 activation, as well as mitochondrial membrane depolarization. There was no stretch-induced increase in caspase-9 activation (Fig. 6A,

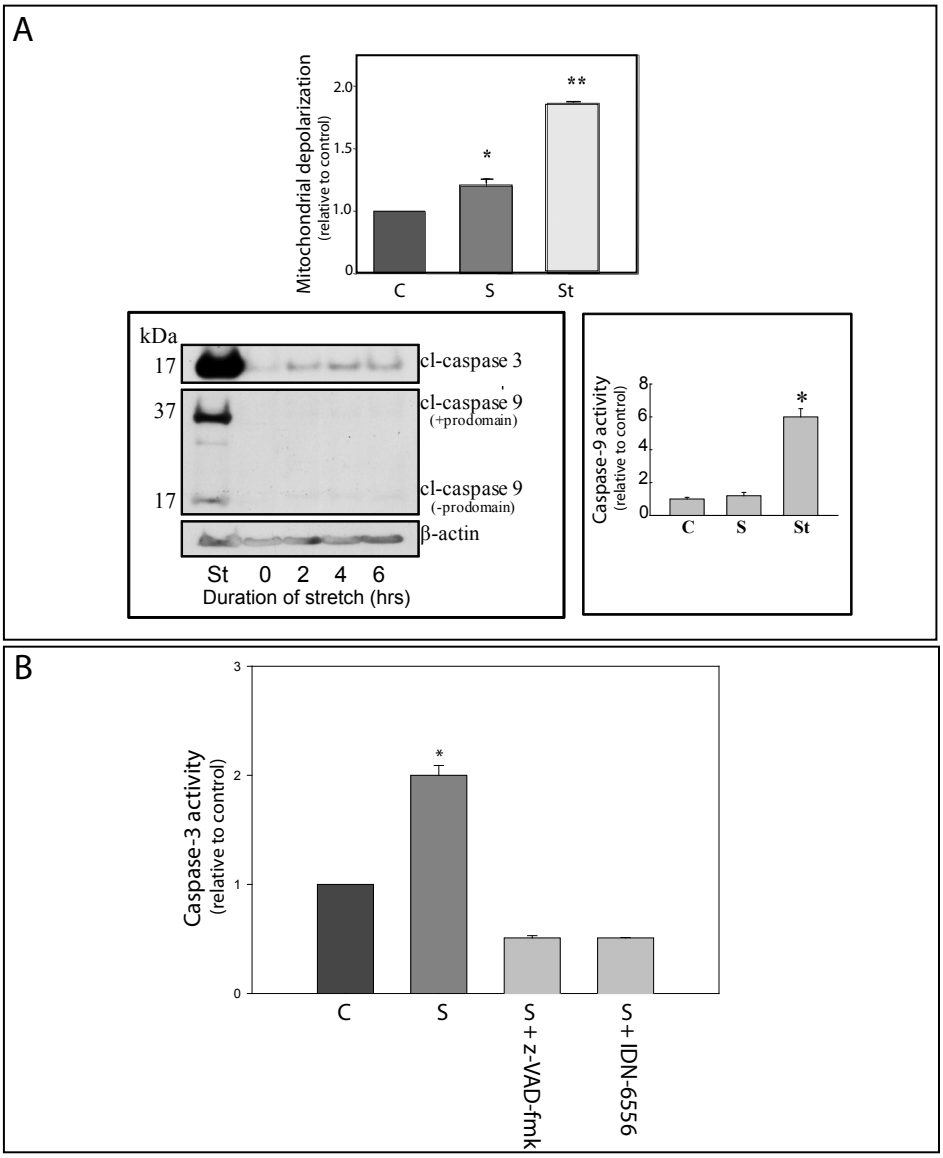

Figure 6. Stretch effect on mitochondrial membrane permeability, caspase -3 and -9 activity. (**A**) Mitochondrial membranes were depolarized but caspase-9 (in contrast to caspase-3) was not activated (bottom panel left: cleaved caspase-3 and -9 formation; bottom panel right: caspase-9 activity) in fetal lung epithelial cells subjected to continuous 17% cyclic stretch for 6 hours. (**B**) Universal casapase inhibitors, z-VAD-fmk and IDN-6556, abrogated 17% stretch-induced caspase-3 activation in rat fetal lung lung epithelial cells. C = static control; S = stretch; St = staurosporine positive control; *$p < 0.05$.

bottom panels), suggesting no or minimal activation of the mitochondrial (intrinsic) death pathway fluorescence (JC-1 monomers) when compared to static control cells, suggesting some by stretch. There was a small, shift in stretched FLDE cells from red

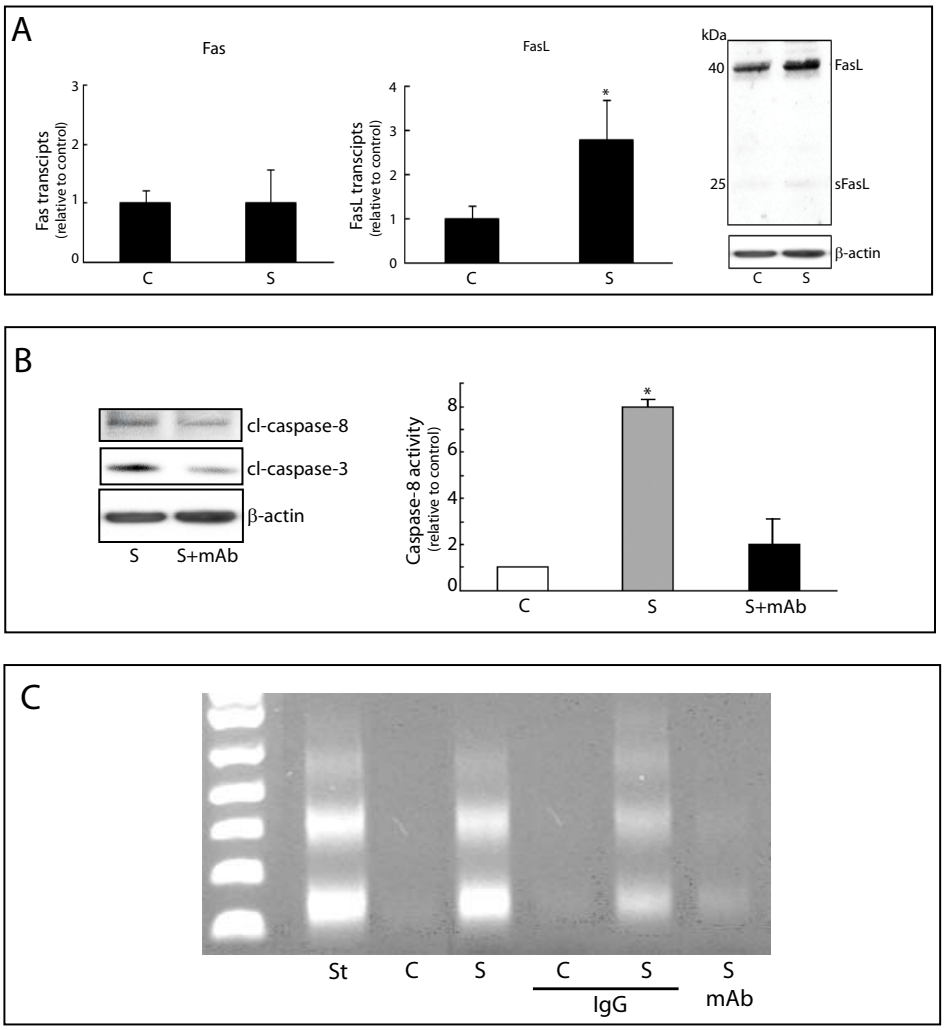

Figure 7. Stretch-induced apoptosis in fetal lung epithelial cells is mediated via FasL. (**A**) FasL, but not Fas, expression is increased in fetal lung epithelial cells subjected to 6 hours of 17% stretch. Neutralizing FasL monoclonal antibodies decrease cleaved caspase-3 and -8 content and caspase-8 activity (**B**) as well as DNA fragmentation (**C**) in fetal lung epithelial cells subjected to 17% stretch. C = static control; S = stretch; St = staurosporine positive control; IgG = antibody control, mAb = monoclonal antibody; *$p < 0.05$.

fluorescence (JC-1 aggregates) to green mitochondrial membrane depolarization in cells subjected to stretch (Figure 6A, top panel), but insufficient to activate caspase-9. Both universal caspase inhibitors ((*ZVAD-fmk and IDN6556*) abrogated stretch-induced caspase-3 activation (Fig. 6B).

Stretch-induced Apoptosis – Role of Fas/FasL: FasL mRNA levels were increased in FLDE cells subjected to stretch (Fig. 7A-middle panel) while Fas mRNA expression was

not changed (Fig. 7A-left panel). Western blotting revealed a small increase in FasL protein in stretched FLDE cells (Fig. 7A, right panel), and a neutralizing anti-rat FasL monoclonal antibody attenuated the stretch-induced levels of cleaved caspases-3 and -8 (Figure 7B-left panel) as well as stretch-induced caspase 8 activity (Fig. 7B-right panel) and stretch-induced DNA laddering (Fig. 7C). An IgG control had no effect (not shown).

DISCUSSION

Apoptosis is critical for normal lung development and function (7, 24), and plays a vital role in remodelling of lung tissue by clearing excess epithelial and mesenchymal cells after injury (25-26). Dysfunctional apoptotic mechanisms likely contribute to the pathophysiology of many lung diseases, including BPD. Mechanical ventilation is a well-recognized risk factor for development of BPD (27-29) and, in the present study, we demonstrate that mechanical ventilation of a newborn rat for 24 h with moderate V_T and in the absence of supplemental O_2 increases apoptosis of alveolar type II cells *via* activation of the FasL/Fas (extrinsic apoptotic) pathway.

Several *in vitro* studies have shown that mechanical stress stimulates apoptosis in isolated lung cells (30-32). Stretch-induced apoptosis *in vivo* was first demonstrated in fetal rabbits after tracheal occlusion (a major lung stretch stimulus) (33), and a recent report demonstrated that mechanical ventilation of newborn mice for 24 h led to a 5-fold increase in the number of apoptotic lung cells (10), and in keeping with the current data, a concomitant increase in cleaved caspase-3 protein (10). The number of caspase-3 positive cells, mainly interstitial cells, was also increased after *in utero* ventilation of fetal sheep for 6 hours (34). In both studies, ventilation-induced apoptosis coincided with a significant reduction in alveolarization (10, 34), in agreement with our findings using the newborn rat model (15). The death pathway(s) by which mechanical ventilation reduces alveolarization were not investigated. In the present study, we demonstrated that the extrinsic pathway (caspase-8 activation) was predominantly activated by mechanical stretch (mimicking moderate/high V_T ventilation) in fetal lung epithelial cells, resulting in activation of effector caspase-3 and -7 and subsequent DNA fragmentation. In contrast, the intrinsic pathway (caspase-9 activation) appeared not to contribute to stretch-induced apoptosis. The 17% cyclic stretch regimen increased apoptotic activity of fetal lung epithelial cells and not in fetal lung fibroblasts. In contrast, mechanical stretch simulating fetal breathing movements (FBM: 5% elongation at intervals of 60 cycles per min, 15 min per h) stimulated apoptosis of rat lung fibroblasts day 19 (canalicular stage), but not epithelial cells, when compared to control (non-stretched) cells (31). Thinning of the mesenchyme is essential during

this stage of lung development to create a close link between the capillary network and airway epithelium (35) and mesenchymal apoptosis during the canalicular stage of lung development has been reported previously (6-7, 36). These observations and our present findings suggest that during normal development, physiological stretch initiates apoptosis in fibroblasts that contributes to structural alignment in the canalicular stage; in contrast, during mechanical ventilation, pathological stretch initiates apoptosis in epithelial cells that contributes to lung injury and abnormal development. In support of this is the observation that pathological (20% cyclic stretch) increased apoptosis and cell death of rat fetal (day 19) type II epithelial cells (31, 37).

In adults with ARDS epithelial cell apoptosis appears to be induced by activation of the FasL/Fas pathway (38-40). In the setting of an inflammatory response, secreted FasL leads to lung epithelial cell apoptosis by binding to and activating Fas antigen in humans (41) and in newborn mice (42). In the present study, ventilation-induced apoptosis was accompanied by an increase in FasL expression in epithelial cells and stretch-induced FasL/Fas pathway activation was confirmed in fetal distal lung epithelial cells. A 17% cyclic stretch of these cells increased the expression of FasL, but not Fas, and neutralizing anti-rat FasL monoclonal antibody reduced the stretch-increased levels of cleaved-caspases 3, 7 and 8. These findings suggest that mechanical ventilation and cell stretch activate the FasL/Fas pathway by increased FasL expression, leading to activation of upstream initiator caspase-8, which in turn activates effector caspase-3 and -7. Alternatively, it is possible that mechanical stretch may increase the proteolytic release of soluble FasL from the membrane, thereby promoting binding to Fas-bearing cells and inducing apoptosis.

Together with the up-regulation of FasL, we found an increase of Egr-1 expression in alveolar type II cells of newborn rats subjected to prolonged mechanical ventilation. Previous *in vivo* studies using adult rats (21) and mice (43) and *in vitro* studies using fetal rat lung epithelial cells (44) have demonstrated that Egr-1 expression is up-regulated in response to mechanical stress while its down regulation by carbon monoxide attenuates ventilator-induced lung injury (43). Thus Egr-1 is an important pro-inflammatory transcriptional regulator that coordinates pro-inflammatory responses (45-46), including transcription of genes involved in growth and apoptosis (46), including FasL (47). The increased expression of both Egr-1 and FasL in alveolar type II cells after ventilation suggest that Egr-1 may activate the extrinsic caspase pathway *via* upregulation of FasL. Another possible mediator is interleukin (IL)-8, and it is known that Egr-1 regulates IL-8 expression (48) and that elevated levels of IL-8 induce apoptosis *via* increased FasL expression (49). Preterm infants developing BPD do, indeed, have elevated serum concentrations of interleukins, including IL-8 (50-51).

Only a few studies have investigated the effect of ventilation on apoptosis in infants with BPD. In preterm infants (gestational wk 24-37) elevated apoptotic activity was

found in bronchiolar and epithelial cells, but not in cells of mesenchymal origin (52). In lung sections from ventilated preterm infants delivered at 22-36 weeks the number of apoptotic epithelial cells was significantly greater than in the lungs of stillborn foetuses of similar gestational age (53), but in contrast to our experiments, these infants were also treated with oxygen. While newborn rats have immature lungs at birth, they do not need mechanical ventilation to survive and do not lack surfactant. However, rat lungs at birth have a saccular appearance (as in preterm infants of 24-30 wks) and alveolarization is an exclusively postnatal (between P4 and P21) event, which makes the newborn rat a suitable model of the infant population developing BPD.

In summary, our findings suggest a central role for the FasL-dependent pathway in ventilation -induced apoptosis of alveolar type II cells which may have therapeutic potential for preventing or treatment of BPD.

REFERENCES

1. Ramanathan R. Optimal ventilatory strategies and surfactant to protect the preterm lungs. Neonatology. 2008;93(4):302-8.
2. Davis PG, Morley CJ, Owen LS. Non-invasive respiratory support of preterm neonates with respiratory distress: continuous positive airway pressure and nasal intermittent positive pressure ventilation. Semin Fetal Neonatal Med. 2009 Feb;14(1):14-20.
3. Morley CJ, Davis PG, Doyle LW, Brion LP, Hascoet JM, Carlin JB. Nasal CPAP or intubation at birth for very preterm infants. N Engl J Med. 2008 Feb 14;358(7):700-8.
4. Lukkarinen HP, Laine J, Kaapa PO. Lung epithelial cells undergo apoptosis in neonatal respiratory distress syndrome. Pediatr Res. 2003 Feb;53(2):254-9.
5. May M, Strobel P, Preisshofen T, Seidenspinner S, Marx A, Speer CP. Apoptosis and proliferation in lungs of ventilated and oxygen-treated preterm infants. Eur Respir J. 2004 Jan; 23(1):113-21.
6. Kresch MJ, Christian C, Wu F, Hussain N. Ontogeny of apoptosis during lung development. Pediatr Res. 1998 Mar;43(3):426-31.
7. Scavo LM, Ertsey R, Chapin CJ, Allen L, Kitterman JA. Apoptosis in the development of rat and human fetal lungs. Am J Respir Cell Mol Biol. 1998 Jan;18(1):21-31.
8. Schittny JC, Djonov V, Fine A, Burri PH. Programmed cell death contributes to postnatal lung development. Am J Respir Cell Mol Biol. 1998 Jun;18(6):786-93.
9. De Paepe ME, Mao Q, Embree-Ku M, Rubin LP, Luks FI. Fas/FasL-mediated apoptosis in perinatal murine lungs. Am J Physiol Lung Cell Mol Physiol. 2004 Oct;287(4):L730-42.
10. Mokres LM, Parai K, Hilgendorff A, Ertsey R, Alvira CM, Rabinovitch M, et al. 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. Jan; 298(1):L23-35.
11. Wajant H. The Fas signaling pathway: more than a paradigm. Science. 2002 May 31; 296(5573):1635-6.
12. De Paepe ME, Rubin LP, Jude C, Lesieur-Brooks AM, Mills DR, Luks FI. Fas ligand expression coincides with alveolar cell apoptosis in late-gestation fetal lung development. Am J Physiol Lung Cell Mol Physiol. 2000 Nov;279(5):L967-76.
13. De Paepe ME, Mao Q, Luks FI. Expression of apoptosis-related genes after fetal tracheal occlusion in rabbits. J Pediatr Surg. 2004 Nov;39(11):1616-25.
14. De Paepe ME, Gundavarapu S, Tantravahi U, Pepperell JR, Haley SA, Luks FI, et al. Fas-ligand-induced apoptosis of respiratory epithelial cells causes disruption of postcanalicular alveolar development. Am J Pathol. 2008 Jul;173(1):42-56.
15. Kroon AA, Wang J, Kavanagh B, Huang Z, Kuliszewski M, van Goudoever JB, et al. Prolonged mechanical ventilation induces cell cycle arrest in newborn rat lung. PLoS One. 2011;6(2):e16910.
16. Caniggia I, Tseu I, Han RN, Smith BT, Tanswell K, Post M. Spatial and temporal differences in fibroblast behavior in fetal rat lung. Am J Physiol. 1991 Dec;261(6 Pt 1):L424-33.
17. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976 May 7;72: 248-54.

18. Yi M, Jankov RP, Belcastro R, Humes D, Copland I, Shek S, et al. Opposing effects of 60% oxygen and neutrophil influx on alveologenesis in the neonatal rat. Am J Respir Crit Care Med. 2004 Dec 1;170(11):1188-96.
19. Roth-Kleiner M, Ridsdale R, Cao L, Kuliszewski M, Tseu I, McKerlie C, et al. Lipopolysaccharide exposure modifies high tidal volume ventilation-induced proinflammatory mediator expression in newborn rat lungs. Pediatr Res. 2007 Feb;61(2):191-6.
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.
21. Copland IB, Kavanagh BP, Engelberts D, McKerlie C, Belik J, Post M. Early changes in lung gene expression due to high tidal volume. Am J Respir Crit Care Med. 2003 Nov 1;168(9): 1051-9.
22. Arora S, Wang Y, Jia Z, Vardar-Sengul S, Munawar A, Doctor KS, et al. Egr1 regulates the coordinated expression of numerous EGF receptor target genes as identified by ChIP-on-chip. Genome Biol. 2008;9(11):R166.
23. Ngiam N, Peltekova V, Engelberts D, Otulakowski G, Post M, Kavanagh BP. Early growth response-1 worsens ventilator-induced lung injury by up-regulating prostanoid synthesis. Am J Respir Crit Care Med. 2010 May 1;181(9):947-56.
24. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972 Aug;26(4):239-57.
25. Sherrill DL, Camilli A, Lebowitz MD. On the temporal relationships between lung function and somatic growth. Am Rev Respir Dis. 1989 Sep;140(3):638-44.
26. Polunovsky VA, Chen B, Henke C, Snover D, Wendt C, Ingbar DH, et al. Role of mesenchymal cell death in lung remodeling after injury. J Clin Invest. 1993 Jul;92(1):388-97.
27. Bhandari A, Bhandari V. Pathogenesis, pathology and pathophysiology of pulmonary sequelae of bronchopulmonary dysplasia in premature infants. Front Biosci. 2003 May 1; 8:e370-80.
28. Lyon A. Chronic lung disease of prematurity. The role of intra-uterine infection. Eur J Pediatr. 2000 Nov;159(11):798-802.
29. Saugstad OD. Chronic lung disease: the role of oxidative stress. Biol Neonate. 1998 Sep; 74 Suppl 1:21-8.
30. Edwards YS, Sutherland LM, Power JH, Nicholas TE, Murray AW. Cyclic stretch induces both apoptosis and secretion in rat alveolar type II cells. FEBS Lett. 1999 Apr 1;448(1): 127-30.
31. Sanchez-Esteban J, Wang Y, Cicchiello LA, Rubin LP. Cyclic mechanical stretch inhibits cell proliferation and induces apoptosis in fetal rat lung fibroblasts. Am J Physiol Lung Cell Mol Physiol. 2002 Mar;282(3):L448-56.
32. Hammerschmidt S, Kuhn H, Grasenack T, Gessner C, Wirtz H. Apoptosis and necrosis induced by cyclic mechanical stretching in alveolar type II cells. Am J Respir Cell Mol Biol. 2004 Mar;30(3):396-402.
33. De Paepe ME, Johnson BD, Papadakis K, Luks FI. Lung growth response after tracheal occlusion in fetal rabbits is gestational age-dependent. Am J Respir Cell Mol Biol. 1999 Jul; 21(1):65-76.
34. Allison BJ, Crossley KJ, Flecknoe SJ, Davis PG, Morley CJ, Hooper SB. Ventilation and oxygen: dose-related effects of oxygen on ventilation-induced lung injury. Pediatr Res. Mar;67(3):238-43.

35. Roth-Kleiner M, Post M. Similarities and dissimilarities of branching and septation during lung development. Pediatr Pulmonol. 2005 Aug;40(2):113-34.
36. Burri PH. Lung development and pulmonary angiogenesis. In: Gaultier C, Bourbon JR, Post M, eds. Lung Development. New York: Oxford University Press, 1999:122-151.1999.
37. Lee HS, Wang Y, Maciejewski BS, Esho K, Fulton C, Sharma S, et al. Interleukin-10 protects cultured fetal rat type II epithelial cells from injury induced by mechanical stretch. Am J Physiol Lung Cell Mol Physiol. 2008 Feb;294(2):L225-32.
38. Albertine KH, Soulier MF, Wang Z, Ishizaka A, Hashimoto S, Zimmerman GA, et al. Fas and fas ligand are up-regulated in pulmonary edema fluid and lung tissue of patients with acute lung injury and the acute respiratory distress syndrome. Am J Pathol. 2002 Nov; 161(5):1783-96.
39. Matute-Bello G, Liles WC, Steinberg KP, Kiener PA, Mongovin S, Chi EY, et al. Soluble Fas ligand induces epithelial cell apoptosis in humans with acute lung injury (ARDS). J Immunol. 1999 Aug 15;163(4):2217-25.
40. Perl M, Chung CS, Perl U, Lomas-Neira J, de Paepe M, Cioffi WG, et al. Fas-induced pulmonary apoptosis and inflammation during indirect acute lung injury. Am J Respir Crit Care Med. 2007 Sep 15;176(6):591-601.
41. Mizuta M, Nakajima H, Mizuta N, Kitamura Y, Nakajima Y, Hashimoto S, et al. Fas ligand released by activated monocytes causes apoptosis of lung epithelial cells in human acute lung injury model in vitro. Biol Pharm Bull. 2008 Mar;31(3):386-90.
42. de Paepe ME, Haley SA, Lacourse Z, Mao Q. Effects of Fas-ligand overexpression on alveolar type II cell growth kinetics in perinatal murine lungs. Pediatr Res. 2010 Jul;68(1): 57-62.
43. Hoetzel A, Dolinay T, Vallbracht S, Zhang Y, Kim HP, Ifedigbo E, et al. Carbon monoxide protects against ventilator-induced lung injury via PPAR-gamma and inhibition of Egr-1. Am J Respir Crit Care Med. 2008 Jun 1;177(11):1223-32.
44. Copland IB, Post M. Stretch-activated signaling pathways responsible for early response gene expression in fetal lung epithelial cells. J Cell Physiol. 2007 Jan;210(1):133-43.
45. Yan SF, Fujita T, Lu J, Okada K, Shan Zou Y, Mackman N, et al. Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. Nat Med. 2000 Dec;6(12):1355-61.
46. Ngiam N, Post M, Kavanagh BP. Early growth response factor-1 in acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2007 Nov;293(5):L1089-91.
47. Kavurma MM, Khachigian LM. Signaling and transcriptional control of Fas ligand gene expression. Cell Death Differ. 2003 Jan;10(1):36-44.
48. Ma J, Ren Z, Ma Y, Xu L, Zhao Y, Zheng C, et al. Targeted knockdown of EGR-1 inhibits IL-8 production and IL-8-mediated invasion of prostate cancer cells through suppressing EGR-1/NF-kappaB synergy. J Biol Chem. 2009 Dec 11;284(50):34600-6.
49. Gangadharan C, Thoh M, Manna SK. Late phase activation of nuclear transcription factor kappaB by doxorubicin is mediated by interleukin-8 and induction of apoptosis via FasL. Breast Cancer Res Treat. 2010 Apr;120(3):671-83.
50. Paananen R, Husa AK, Vuolteenaho R, Herva R, Kaukola T, Hallman M. Blood cytokines during the perinatal period in very preterm infants: relationship of inflammatory response and bronchopulmonary dysplasia. J Pediatr. 2009 Jan;154(1):39-43 e3.

51. Ambalavanan N, Carlo WA, D'Angio CT, McDonald SA, Das A, Schendel D, et al. Cytokines associated with bronchopulmonary dysplasia or death in extremely low birth weight infants. Pediatrics. 2009 Apr;123(4):1132-41.
52. Hargitai B, Szabo V, Hajdu J, Harmath A, Pataki M, Farid P, et al. Apoptosis in various organs of preterm infants: histopathologic study of lung, kidney, liver, and brain of ventilated infants. Pediatr Res. 2001 Jul;50(1):110-4.
53. May M, Marx A, Seidenspinner S, Speer CP. Apoptosis and proliferation in lungs of human fetuses exposed to chorioamnionitis. Histopathology. 2004 Sep;45(3):283-90.

Chapter 6

Prolonged Mechanical Ventilation Induces Cell Cycle Arrest in Newborn Rat Lung

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ABSTRACT

Rationale: The molecular mechanism(s) by which mechanical ventilation disrupts alveolar development, a hallmark of bronchopulmonary dysplasia, is unknown. *Objective*: To determine the effect of 24 hours (s) of mechanical ventilation on lung cell cycle regulators, cell proliferation and alveolar formation in newborn rats. *Methods:* Seven-day old rats were ventilated with room air for 8, 12 and 24 h using relatively moderate tidal volumes (8.5 $mL.kg^{-1}$). *Measurement and Main Results*: Ventilation for 24 h decreased the number of elastin-positive secondary crests and increased the mean linear intercept, indicating arrest of alveolar development. Proliferation (assessed by BrdU incorporation) was halved after 12 h of ventilation and completely arrested after 24 h. Cyclin D1 and E1 mRNA and protein levels were decreased after 8-24 h of ventilation, while that of $p27^{Kip1}$ was significantly increased. Mechanical ventilation for 24 h also increased levels of $p57^{Kip2}$, decreased that of $p16^{INK4a}$, while the levels of $p21^{Waf/Cip1}$ and $p15^{INK4b}$ were unchanged. Increased $p27^{Kip1}$ expression coincided with reduced phosphorylation of $p27^{Kip1}$ at Thr^{157}, Thr^{187} and Thr^{198} ($p<0.05$), thereby promoting its nuclear localization. Similar -but more rapid- changes in cell cycle regulators were noted when 7-day rats were ventilated with high tidal volume (40 $mL.kg^{-1}$) and when fetal lung epithelial cells were subjected to a continuous (20% elongation) cyclic stretch. *Conclusion*: This is the first demonstration that prolonged (24 h) of mechanical ventilation causes cell cycle arrest in newborn rat lungs; the arrest occurs in G_1 and is caused by increased expression and nuclear localization of Cdk inhibitor proteins ($p27^{Kip1}$, $p57^{Kip2}$) from the Kip family.

INTRODUCTION

Introduction of more gentle ventilation strategies -together with surfactant replacement and antenatal corticosteroids- has improved the survival rate of very premature infants. In parallel, the number of infants with 'new' bronchopulmonary dysplasia (BPD) (1) has also increased. Currently, infants born at ≤26 weeks of gestation are at the greatest risk of developing such 'new' BPD (2), a syndrome of arrested lung development with fewer and larger alveoli and dysmorphic vasculature (3). BPD can no longer be considered only a pediatric disease because the substantial lung-function abnormalities -and significant symptoms- persist into adulthood (4-6). The pathogenesis of BPD is incompletely understood and its treatment is empirical, but mechanical ventilation remains a major risk factor.

Lung development between 24-32 weeks of gestation is characterized by extensive vasculogenesis within the developing terminal saccules, followed by secondary crest formation as well as interstitial extracellular matrix loss and remodeling (7). This tissue remodeling requires well-coordinated regulation of cell proliferation and apoptosis. Recent studies have shown that prolonged mechanical ventilation increases apoptosis and impairs alveolar septation in newborn mice (8), however the effect of mechanical ventilation on lung cell growth is mostly unknown. *In vitro* studies have demonstrated that mechanical stretch (5% elongation, 60 cycles per min, 15 min/h for 24 h) and oxygen (95%) can inhibit lung cell proliferation (9-10), but molecular mechanisms are yet to be determined. Cell proliferation is a precisely coordinated set of events regulated by interaction of gene products that activate or suppress cell cycle progression. A series of cyclins and cyclin-dependent kinases (Cdk) act in concert to drive the cycle forward through the G_1, S and G_2/M phases (11). In mammalian cells, G_1/S transition is an important checkpoint in the cell cycle. Entry into the cell cycle is initiated by mitogen-stimulated expression of D-type cyclins which activate Cdk4/6. Shortly thereafter, cyclin E expression is increased and cyclin E-Cdk2 complexes are formed, promoting entry into the S phase (12). While cyclin-Cdk complexes positively drive progression of the cell cycle, Cdk inhibitors (CKI) negatively regulate progression by binding to and inactivating cyclin–Cdks (13). There are two distinct CKI families in mammalian cells: INK4 proteins, which block the progression of the cell cycle by binding to either Cdk4 or Cdk6 and inhibiting the action of cyclin D; and, Cip/Kip proteins that inhibit a broader spectrum of cyclin-Cdk complexes (14-16).

In this study we determined the effect of prolonged (24 h) mechanical ventilation on lung cell cycle regulators, proliferation and alveolar formation in a newborn rat model (17). We hypothesized that continuous cyclic (over)stretching of the primitive airsacs would adversely affect proliferation of lung cells by influencing cell cycle regulators.

METHODS

Ethics statement: The study was conducted according to the guidelines of the Canadian Council for Animal Care and with approval of the Animal Care Review Committee of the Hospital for Sick Children (protocol #7217).

Animal preparation: Timed pregnant Wistar rats (Charles River, Oakville, Quebec, Canada) were allowed to deliver and immediately afterwards litters were reduced to 10 pups. Newborn rat pups were anesthetized by *i.p.* injection of 30 mg kg^{-1} pentobarbital and a tracheotomy was performed. The trachea was cannulated with a 1 cm 19G cannula and connected to a rodent ventilator (FlexiVent Scireq, Montreal, PQ). Spontaneously breathing, non-ventilated, littermates served as sham controls. One pup per litter was ventilated and a littermate was used as non-ventilated control. Isoflurane was used as general anesthesia during the ventilation period and 0.9% saline (100 $ml.kg^{-1}$/24 h) was administered subcutaneously by continuous infusion with a 27G needle to prevent dehydration. First rat pups at postnatal days 6, 7, 8, 9, 10 and 14 were ventilated to assess lung cell proliferation. For all subsequent experiments 7-day old rat pups were used. Preliminary experiments were performed to determine ventilator settings (18). Starting from a normal respiratory rate for newborn rats (150 bpm), tidal volume was adjusted to achieve normal blood gas values after the ventilation period. Animals were monitored by ECG. Rectal temperature was maintained at 37°C using a thermal blanket, lamp and plastic wrap. At the end of the ventilation period a blood sample from the carotid artery was taken for blood gas analysis prior to euthanasia.

Mechanical ventilation: Rat pups were ventilated with room air and moderate-V_T (8.5 $mL.kg^{-1}$, RR 150 min^{-1}, PEEP 2 cm H_2O) for 8, 12 and 24 h. In a few cases, pups were ventilated for 4 h with high-V_T (40 $mL.kg^{-1}$, RR 30 min^{-1}, PEEP 2 cm H_2O). The 7-day old pups weighed 15.5-18.6 g. Dynamic compliance was estimated every 4 h from data obtained during a single-frequency forced oscillation manoeuvre, using a mathematical model-fitting technique according to the specifications of Scireq Inc. (Montreal, PQ). Two h before completion of ventilation pups were injected *ip* with 50 mg/kg 5-bromo-2-deoxyuridine (BrdU). At completion of ventilation a blood sample was taken from the carotid artery for blood gas analysis and the animals killed by exsanguination. Lung tissues were processed for histology or fresh frozen for molecular/protein analyses.

Histology: After flushing whole lungs were infused in situ with 4% (w/v) paraformaldehyde (PFA) in PBS with a constant pressure of 20 cm H_2O over 5 minutes to have equalized filling pressure over the entire lung. Under these constant pressure conditions the cannula was removed and the trachea immediately ligated. The lungs were excised

and immersed in 4% PFA in PBS overnight, dehydrated in a ethanol/xylene series and embedded in paraffin. Sections of 5 µm were stained with hematoxilin and eosin or stained for elastin using accustain artrazine solution (Sigma, St. Louis MO, USA).

Immunohistochemistry: Following sectioning and antigen retrieval by heating in 10 mM sodium citrate pH 6.0, sections were washed in PBS and endogenous peroxidase was blocked in 3% (v/v) H_2O_2 in methanol. Blocking was done with 5% (w/v) normal goat serum (NGS) and 1% (w/v) bovine serum albumin (BSA) in PBS. Sections were then incubated overnight at 4°C with either 1:50 diluted mouse anti-BrdU (Boehringer Mannheim, Germany) or 1:400 diluted rabbit anti-phospho-histone H3 (Millipore, Billerica, MA, USA) antibodies (Lab Vision Corporation, Fremont, Canada). Biotinylated rabbit anti-mouse IgG or goat anti-rabbit IgG were used as secondary antibodies, respectively. Color detection was performed according to instruction in the Vectastain ABC and DAB kit (Vector Laboratories, Burlingname, CA, USA). All sections were counterstained with hematoxylin. For quantitative analysis, digital images were captured using a Leica digital imaging system at 20× magnification with random sampling of all tissue in an unbiased fashion. Images were captured randomly from 15 non-overlapping fields from each slide, with 3 slides per animal and 4 animals per group.

Morphometric analysis: Digital images were captured from either H&E- or elastin-stained slides with random sampling of all tissue in an unbiased fashion. Images were captured randomly from 15 non-overlapping fields/slide with 3 slides/animal and 4 animals/group. Tissue fraction was calculated from pixel counts of black/white images (19), mean linear intercepts (Lm) were measured and calculated (20) and the number of elastin-positive secondary septa determined.

Western blot analysis: Lung tissues were lysed, protein content measured (21) and aliquots (40 g protein) were subjected to 10% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% (w/v) skim milk in TBST (20mM Tris, 137 mM NaCl, 0.1% Tween 20) membranes were incubated with appropriate primary antibody overnight in 4°C. Because of decreased BrdU incorporation and cyclin D1 and E1 expression, we focused on CKIs inhibiting Cdk-2, -4 and -6 (22). Primary antibodies were rabbit anti-$p15^{INK4B}$ (dilution of 1:500), rabbit anti-$p16^{INK4A}$ (dilution of 1:1000), mouse anti-$p21^{Waf1/Cip1}$ (dilution 1:500), rabbit anti-$p27^{Kip1}$(dilution 1:500) and rabbit anti-$p57^{Kip2}$ (dilution of 1:1000), rabbit anti-cyclin D1 (dilution of 1:1000) (all from Cell Signaling Technology, Danvers, USA) and rabbit anti-cyclin E1 (dilution of 1:1000) (Santa Cruz Biotechnology, Santa Cruz USA). Primary phosphorylated $p27^{Kip1}$ antibodies were rabbit anti-$p27^{Kip1}$ (pThr198) (dilution of 1:400) and rabbit anti-$p27^{Kip1}$ (pSer10)-R (dilution of 1:2000; both from Santa Cruz Biotechnology, Santa Cruz, USA), rabbit

anti-p27^{Kip1} (pThr157) (dilution of 1:300; R&D Systems Inc, Burlington, Canada) and rabbit anti-p27^{Kip1} (pThr187) (dilution of 1:400; Novus Biologicals, Littleton, USA). The next day the membranes were washed TBST and incubated with either horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG (dilution of 1:1000; Cell Signaling Technology, Danvers, USA). After several washes with TBST, protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ, USA). Band densities were quantified using Scion Image software (Version 1.6, National Institutes of Health, Bethesda, MD, USA). Equal protein loading was confirmed by immunoblotting for β-actin of same membrane.

Quantitative RT-PCR: Total RNA was extracted from lung tissues and reverse transcribed [14]. Complementary DNA was amplified for target genes cyclin D1, cyclin E1 and p27 as previously described (17, 19). For relative quantification, polymerase chain reaction signals were compared between groups after normalization using 18S as internal reference. Fold change was calculated (23).

Stretch of epithelial cells isolated from fetal rat lungs: Distal fetal lung epithelial cells (day 19 of gestation) were isolated as previously described (24). Cells were cultured on type-1 collagen-coated BioFlex plates and subjected various durations of cyclic continuous 20% stretch using a FX-4000 Flexercell Strain Unit (Flexercell Int., NC, USA) (25). Neither cell viability (trypan blue exclusion) nor cell attachment was affected by any of the applied stretch regimens. Cell lysates were processed for Western Blotting.

Statistical analysis: Stated otherwise all data are presented as mean ± SD. Data was analyzed using SPSS software version 15 (SPSS Inc, Chicago, IL). Statistical significance ($p < 0.05$) was determined by using one-way ANOVA or Kruskal-Wallis test. *Post hoc* analysis was performed using Duncan's multiple-range test (data presented as mean ± SD) or Mann-Whitney test (data presented as median and interquartile range).

RESULTS

Physiologic data: Blood gases were in the normal range after 8, 12 and 24 h of ventilation (Table 1). Mean airway pressures, peak pressures and delivered V_T remained constant up to 8 h of ventilation (18), but altered slightly after 12 h of ventilation compared to baseline (Table 1). Dynamic compliance of the respiratory system was constant up to 8 h of ventilation (18) decreased after 12 h and remained stable afterwards (Fig. 1). These results are indicative of the impact of 8 h of ventilation that did not subsequently worsen.

Table 1. Blood gas analysis and airway pressures after 8, 12 and 24 h of ventilation

	8 hrs	12 hrs	24 hrs
pH	7.39 ± 0.07	7.29 ± 0.05	7.30 ± 0.05
pCO_2 (mmHg)	44.3 ± 6.4	35.8 ± 6.2	39.4 ± 4.5
pO_2 (mmHg)	83 ± 8.4	87.4 ± 11.2	73.5 ± 11.7
BE (mmol/L)	-4.2 ± 2.3	-3.9 ± 1.6	-5.2 ± 2.1
Ppeak (cm H_2O)	10.9 ± 1.1	12.7 ± 1.1*	13.1 ± 1.3*
Pmean (cm H_2O)	6.3 ± 0.5	7.2 ± 0.5*	7.5 ± 0.6*
PEEP (cm H_2O)	2	2	2
Frequency (breaths/min)	150	150	150
Delivered V_T (ml/kg)	8.9 ± 0.2	8.5 ± 0.4*	8.4 ± 0.2*

Values represent means ± SD, n = 12 animals per group. *p < 0.05 versus values at 0 hrs. Ppeak, peak pressure; Pmean, mean pressure; PEEP, positive-end expiratory pressure.

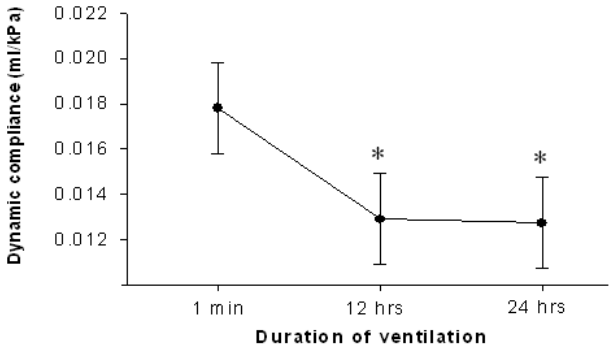

Figure 1. Dynamic compliance during 24 h of mechanical ventilation. Dynamic compliance decreased during first 12 h of ventilation with room air and low/moderate V_T but remained stable during the next 12 h. Data are mean ± SD, n = 12 rat pups per time group. *p < 0.05 prolonged versus 1-min ventilation.

Morphometric analyses: Seven-day old rat pups ventilated for 12 and 24 h had fewer and larger alveoli when compared to the lungs of non-ventilated 8 day-old pups (Fig. 2A). The tissue-to-air ratio corroborated these findings; it decreased after 12 h of ventilation and declined further during the next 12 h of ventilation (Fig. 2B). To quantify alveolar development, we calculated the number of elastin-positive secondary crests per unit area (Fig. 2D). The number of secondary crests -indicating alveolar formation- increased significantly between the 7^{th} and 8^{th} postnatal days in non-ventilated rat pups. The number of secondary crests increased after 12 h of ventilation when compared to day 7 controls. In contrast, the number of secondary crests was significantly lower in pups ventilated for 24 h *vs*. non-ventilated day 8 control pups, even when corrected for tissue fraction. To further evaluate alveolar development, we measured the mean linear intercept (Lm; Fig. 2C). Ventilation increased the Lm after 12 h, and more so after 24 h.

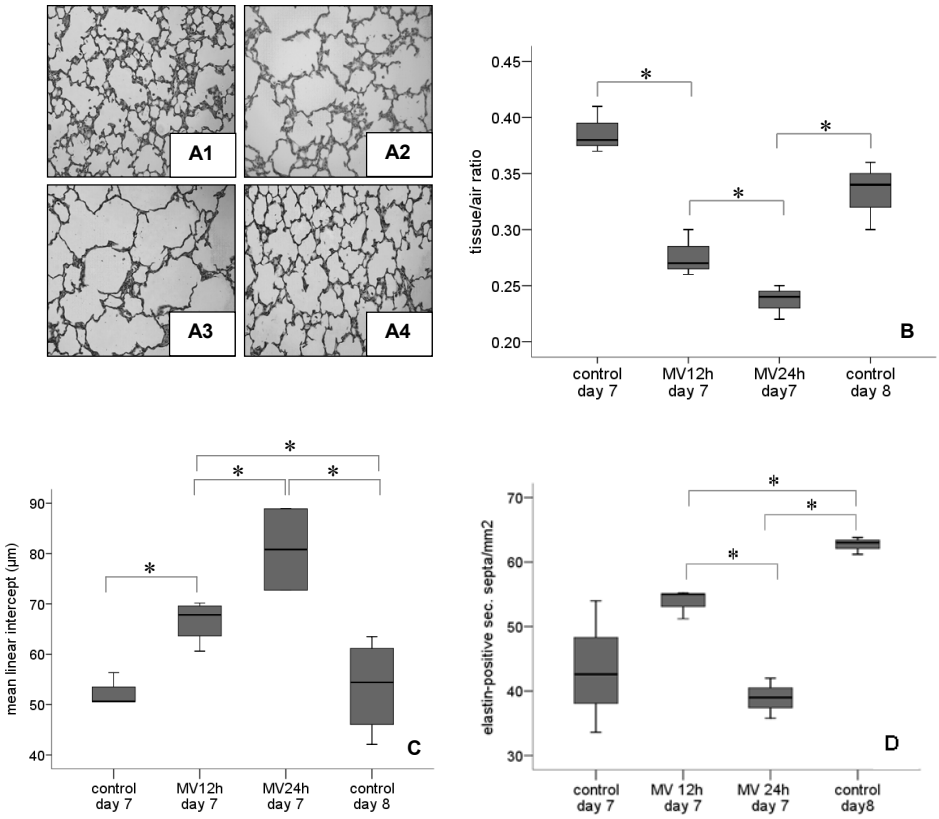

Figure 2. Ventilation inhibits alveolar growth. (A) Histology after mechanical ventilation: (A1) non-ventilated 7-day old rat (A2) 7-day old rat ventilated for 12 h, (A3) 7-day old rat ventilated for 24 h, (A4) non-ventilated 8-day old rat. (B) Mechanical ventilation for 12 and 24 h significantly increased alveolar airspace (reduction in tissue-to-air ratio (A) as well as increase in mean linear intercept (D)) but decreased number of elastin-positive secondary septa (C). Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles, n = 12 rat pups per time group. MV, mechanical ventilation. *$p < 0.05$.

Together the data suggest that during the first 12 h of ventilation alveolar space increases because of hyperinflation while a further increase of alveolar space during the next 12 h of ventilation is in part due to arrest in alveolar development as well as hyperinflation.

Lung cell proliferation: Lung cell proliferation was assessed in non-ventilated *vs*. ventilated rat pups at postnatal days 6, 7, 8, 9, 10 and 14. In non-ventilated rats, the number of proliferating lung cells was greatest at postnatal day 6 (BrdU labelling index: ~12%), which declined gradually to almost undetectable at day 15 (Fig. 3). Ventilation for 24 h clearly inhibited lung cell proliferation in pups of all studied ages (days 6-14). Next, 7-day old rat pups were ventilated for all subsequent experiments. Proliferation

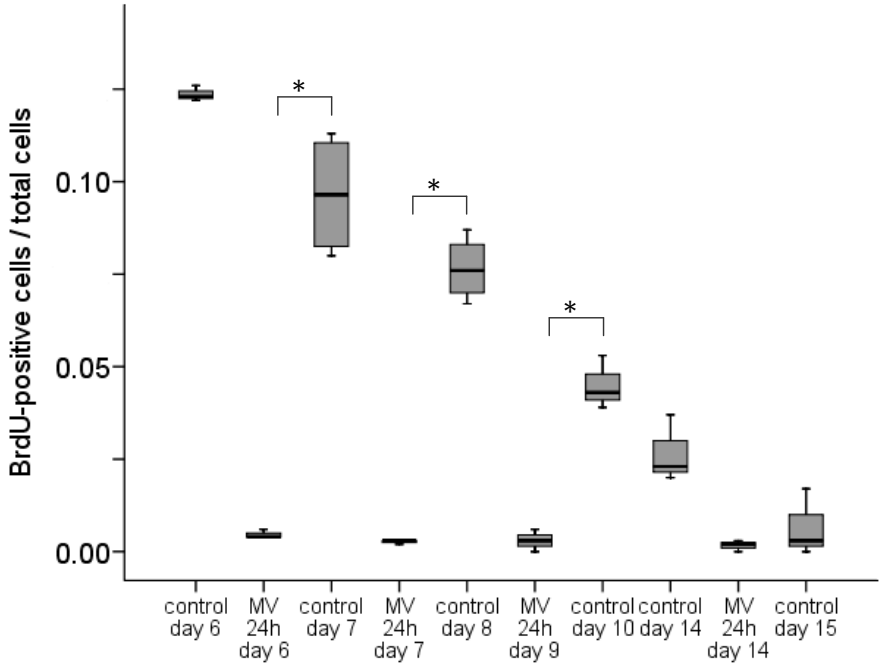

Figure 3. Ventilation inhibits BrdU uptake independent of postnatal age. Although the BrdU labeling index decreased gradually with advancing postnatal gestation, mechanical ventilation for 24 h inhibited cell proliferation at every postnatal age. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles, n=4 rat pups per time group. *p < 0.05.

was not affected by 8 h of ventilation (data not shown) but longer durations of ventilation significantly decreased the number of proliferating cells (Fig. 4 A, B). The ratio of proliferating mesenchymal and epithelial cells did not significantly differ between non-ventilated pups and pups ventilated for 8 and 12 h, respectively (0.73±0.05 vs. 0.65±0.03 and 0.67±0.1). Since a 12 h ventilation decreased the total number of proliferating cells (Fig. 4B) the unchanged ratio suggest that cell proliferation of both tissue layers was equally affected by mechanical ventilation. Hardly any proliferating cells were seen after 24 h of ventilation; in agreement with a reduction in cell proliferation in both tissue layers. The almost total arrest in lung cell proliferation by prolonged (24 h) ventilation was confirmed by anti-PH3 immunochemistry (PH3-positive cells decreased from 8 to 1% of total).

Cell cycle regulators: mRNA levels of lung cyclin D1 and E1 were significantly down-regulated after 8, 12 and 24 h of ventilation while that of $p27^{Kip1}$ was increased (Fig. 5A). Immunoblot (i.e. protein) analysis of lungs ventilated for 24 h confirmed these mRNA changes of cyclin D1, E1 and $p27^{Kip1}$ (Figs. 5B, 5C, 6A). The amount of $p27^{Kip1}$ was 1.5-fold increased after 12 h of ventilation (not shown). Other members of the Cip/

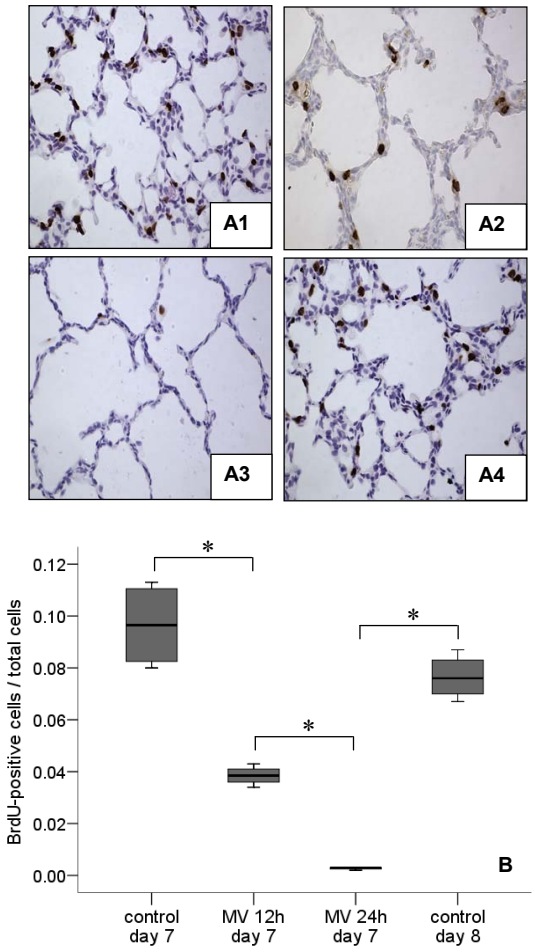

Figure 4. Ventilation inhibits lung cell growth. Immunohistochemistry ((A1) non-ventilated 7-day old rat, (A2) 7-day old rat ventilated for 12 h, (A3) 7-day old rat ventilated for 24 h, (A4) non-ventilated 8-day old rat) illustrates reduction in BrdU uptake (brown color) with duration of ventilation. (B) BrdU labeling index significantly decreased after 12 and 24 h of mechanical ventilation. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles, n=12 rat pups per time group. MV, mechanical ventilation. *$p < 0.05$.

Kip family of CKIs were either increased (p57^{Kip2}, Fig. 6B) or unchanged (p21$^{Waf/Cip1}$, not shown) by 24 h of ventilation. In contrast, CKIs belonging to the INK4 family were either reduced (p16^{INK4a}) or not affected (p15^{INK4b}) by 24 h of ventilation (Fig. 6C, D). p27^{Kip1} can be phosphorylated at different sites, which influences its localization and activity (26). A 12 h ventilation decreased phosphorylation of p27^{Kip1}at Thr^{157} (Fig. 7A) but did not affect phosphorylation of Thr^{198} (not shown). However, mechanical ventilation for 24 h decreased p27^{Kip1} phosphorylation at Thr^{157}, Thr^{187} and Thr^{198}, thereby promot-

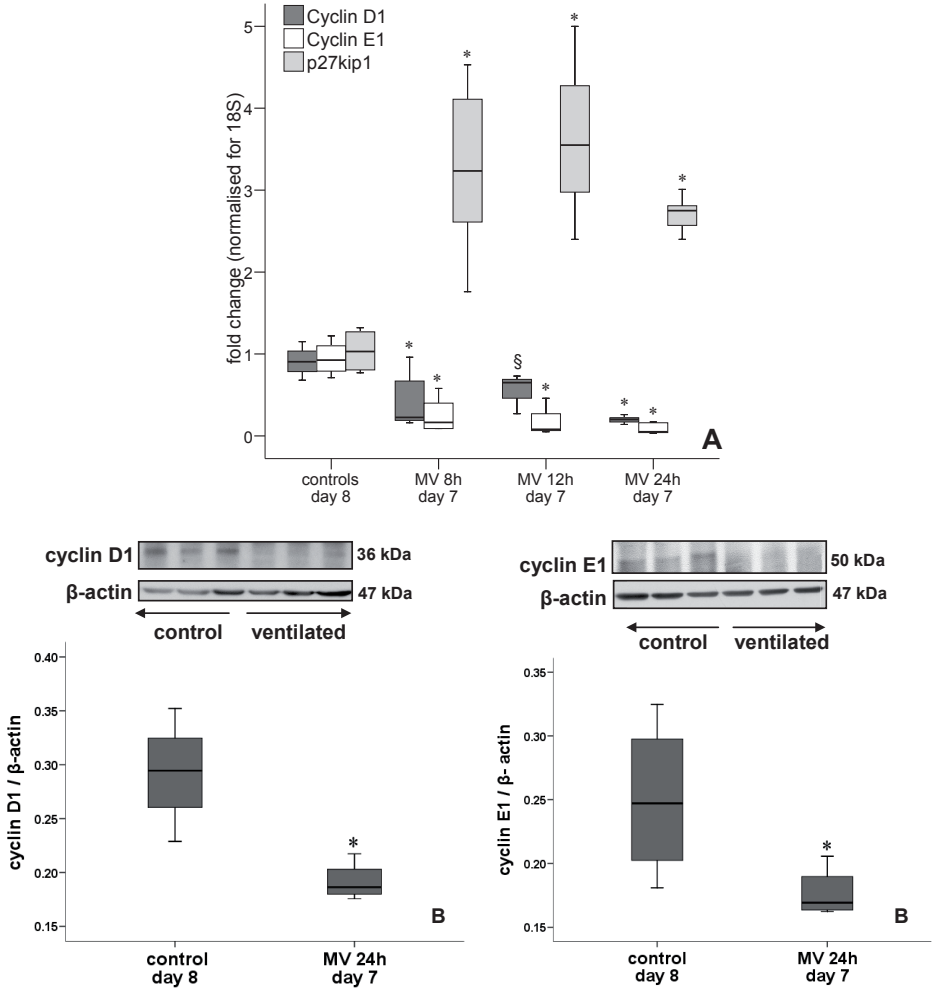

Figure 5. Impact on cyclin D, cyclin E and $p27^{Kip1}$ expression. Mechanical ventilation for 24 h significantly decreased cyclin D1 and E1 mRNA (A) and protein (B and C) levels in lungs of 7-day old rats. In contrast, $p27^{kip1}$ mRNA increased (A). Inserts in (B) and (C) show cyclin D1 (B) and cyclin E1 (C) immunoblots of lung tissue of 7-day old rats ventilated for 24 h and non-ventilated 8-day old rats (controls). Blots were reprobed with β-actin for equal loading and transfer. Medians with 25^{th} and 75^{th} quartiles are shown, bars are 5^{th} and 95^{th} percentiles; qPCR, n=6 rat pups per time group; immunoblot, n=3 rat pups per time group. MV, mechanical ventilation. *$p < 0.05$ versus non-ventilated group, $^{§}p < 0.05$ versus 24h ventilation.

ing stability and nuclear localization (Fig. 7B-D). Similar -but more rapid- changes in cell cycle regulators were noted when 7-day newborn rats were ventilated with high V_T. Although β-actin can be responsive to stretch, no significant differences in β-actin expression was noted between ventilated animals and controls (not shown). High V_T reduced the amount of D1 and D2 cyclins within 1 hour, while that of Cdk inhibitors

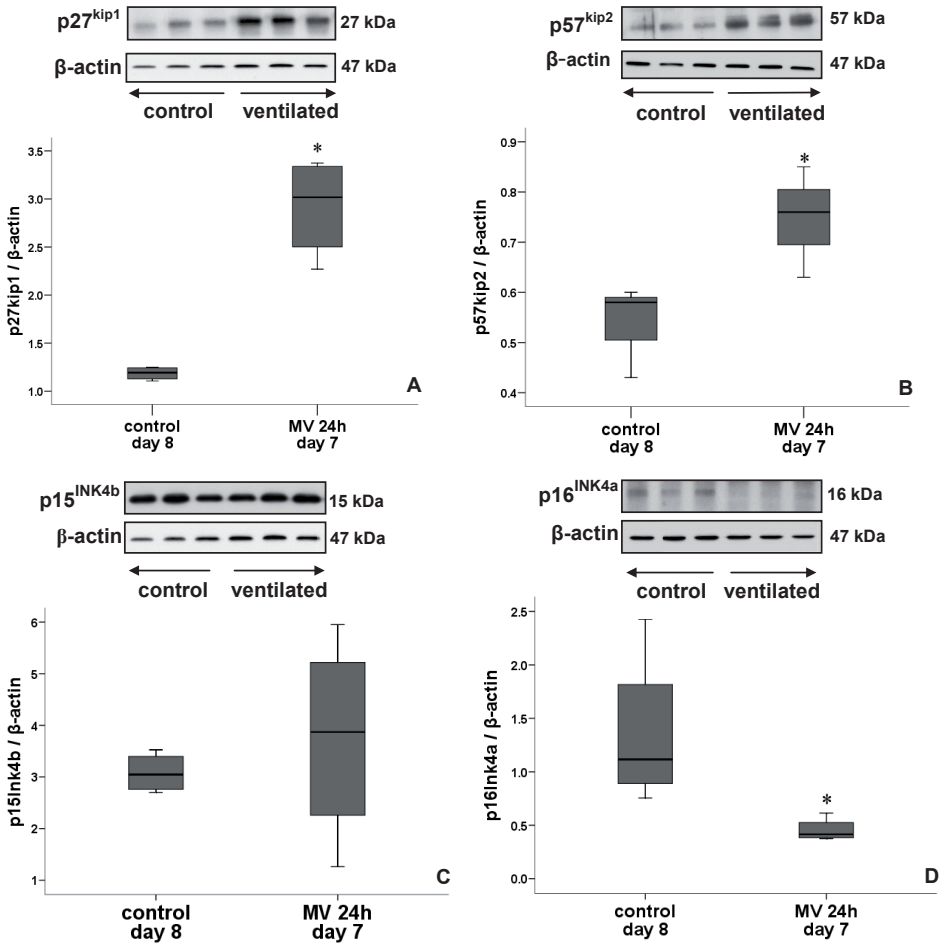

Figure 6. Different effects on Kip and INK proteins. Mechanical ventilation for 24 h significantly increased $p27^{Kip1}$ (A) and $p57^{Kip2}$ (B) protein levels. In contrast, $p16^{INK4a}$ protein (D) was decreased by ventilation while $p15^{INK4b}$ (C) was unchanged. Inserts show immunoblots of lung tissue of 7-day old rats ventilated for 24 h and non-ventilated 8-day old rats (controls). Blots were reprobed with β-actin for equal loading and transfer. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles; n=3 rat pups per time group. *$p < 0.05$ versus non-ventilated group.

$p27^{Kip1}$ and $p57^{Kip2}$ increased (Fig. 8A); in contrast, $p16^{INK4a}$ content was decreased by high-V_T ventilation.

We do not know in which particular tissue layer (epithelium, mesenchyme) these changes occurred *in vivo*, but they at least occur in epithelial cells as subjecting *ex vivo* fetal lung epithelial cells to cyclic continuous 20% stretch resulted in similar patterns of alteration in cell cycle regulators (Fig. 8B).

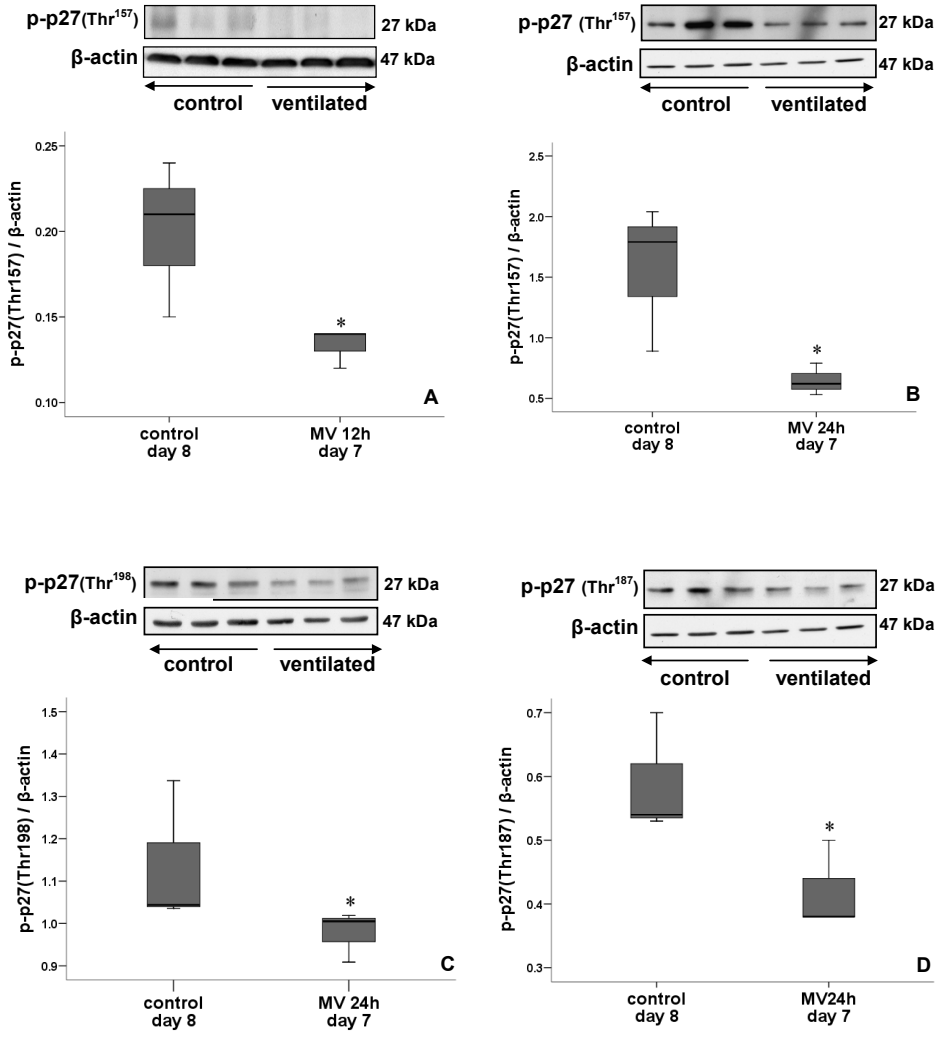

Figure 7. Effect on p27^{kip1} phosphorylation. A 24h-ventilation significantly decreased Thr^{157}-phosphorylated p27^{Kip1} (A), Thr^{187}-phosphorylated p27^{Kip1} (B) and Thr^{198}-phosphorylated p27^{Kip1} (C). Phosphorylation of threonine 157 was already reduced after 12 h of ventilation (D). Inserts show immunoblots of lung tissue of 7-day old rats ventilated for 24 h and non-ventilated 8-day old rats (controls Blots were reprobed with β-actin for equal loading and transfer. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles; n=3 rat pups per time group. *$p < 0.05$ versus non-ventilated group.

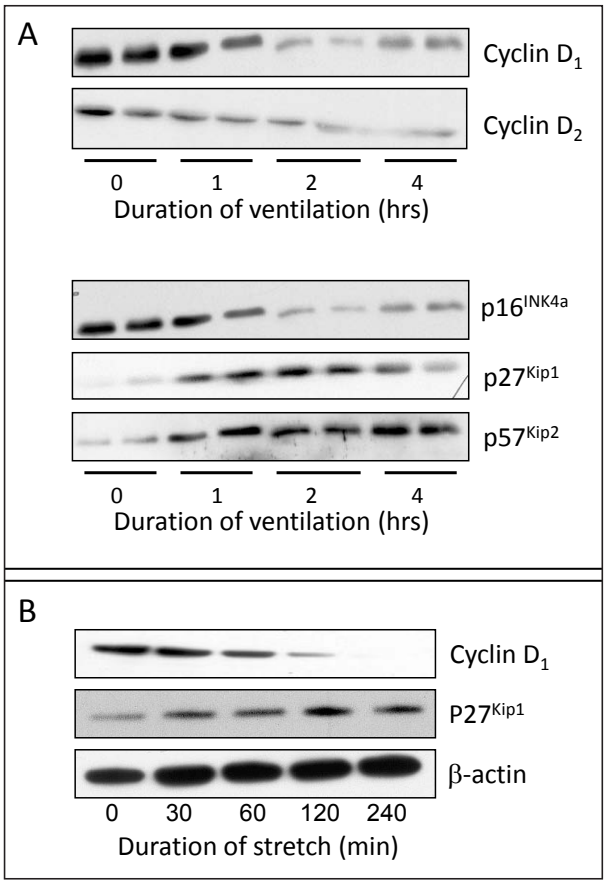

Figure 8. Effect of high tidal volume ventilation and in vitro stretch. High V_T ventilation of 7-day old rat lungs (A) and a continuous cyclic 20% stretch of fetal lung epithelial cells (B) rapidly decreased type-D cyclins and $p^{16INK4a}$ while increasing Kip proteins, in particular $p27^{Kip1}$. Blots were reprobed with β-actin for equal loading and transfer. Representative blots of 2 experiments carried out in duplicate (A) or 3 experiments (B).

DISCUSSION

The hallmark of 'new' BPD is arrested alveolarization (1), but the molecular and cellular basis of the alveolar arrest remains mostly unknown. Alveolarization occurs as the immature saccules, which form the lung parenchyma at birth, are subdivided into smaller units by the formation and extension of secondary septa; new tissue ridges are lifted off the existing primary septa and grow in a centripetal direction into the airspaces. This process, called septation, is mainly postnatal (human: 36 weeks-infancy; rat: Pnd5–Pnd21) (7, 27). Before septation of the air spaces starts, the lung expands for a short period of time, and the cells of the inter-airway walls actively proliferate, peaking at day

5 in rats and steadily declining thereafter (28). This active cell proliferation takes place just at the beginning of the septation of the distal airways. With the use of a newborn rat model (18) we demonstrate here that mechanical ventilation for 24 h with room air and moderate V_T results in cell cycle arrest, and reduced alveolar septation. This ventilation strategy (room air and moderate VT) was chosen to avoid/minimize lung injury.

In rats, lungs at birth have a saccular appearance and alveolarization is an exclusively postnatal (between P4 and P21) event, which makes this model relevant to the infant population developing BPD. However, major differences exist between mechanically ventilated newborn rats and premature born infants. Newborn rats have an immature lung architecture at birth, but they do not need mechanical ventilation to survive (likely due to differences in airway structure, with large airways extending almost to the lung periphery and quickly reducing in diameter to the alveoli) and they do not lack surfactant. Infants with BPD demonstrate interstitial thickening that may partly be due to fibroproliferation while in rat pups mechanical ventilation of 24 h caused cell arrest in both mesenchymal and epithelial cell layers. Despite these differences, our results suggest that the observed cell cycle arrest is due to increased expression of two CKIs (i.e. $p27^{Kip1}$ and $p57^{Kip2}$) that are members of the Cip/Kip family; the other member, $p21^{waf/Cip1}$, was not affected by 24 h of mechanical ventilation.

Knock-in mouse models have shown that $p27^{Kip1}$ and $p57^{Kip2}$ are interchangeable *in vivo* (29), suggesting similar mechanisms of regulation. Mechanical strain has been recognized as playing an important role in the regulation of fetal lung cell proliferation. Indeed the stimulatory effect of mechanical stretch (i.e. increased intratracheal pressure) on fetal lung growth has been extensively studied in tracheal occlusion (TO) models (30-31), where the number of proliferating alveolar type II cells significantly increased. Fetal sheep, exposed to TO during the alveolar stage of lung development, showed an increase in alveolar type II cells between days 2-4 after TO (31). This proliferative response of fetal lung cells to strain has also been demonstrated *in vitro*. Intermittent cyclic 5% stretching (simulating normal fetal breathing movements) of distal fetal rat lung cells (epithelial cells and fibroblasts) increased cell proliferation (32). However, a continuous cyclic 20% stretch (simulating mechanical ventilation (33)) for 24 h inhibited fetal lung cell proliferation (unpublished results), in agreement with our *in vivo* findings of a proliferative arrest after 24 h of mechanical ventilation. In the present study, continuous cyclic 20% stretch of fetal lung epithelial cells caused similar alterations in cell cycle regulators as observed in mechanically ventilated newborn lungs *in vivo*, namely increased levels of $p27^{Kip1}$ and a decrease in the amount of cyclin D1. CKI members of the Cip/Kip family ($p21^{WAF1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) preferentially inhibit cyclin-Cdk2 complexes (16). How mechanical stretch influences CKIs is unknown. In many cancers, the ras/raf/mitogen activated protein kinase (MAPK) pathway increases $p27^{Kip1}$ proteolysis while downstream effectors of the PI-3K pathway such as protein

kinase B (also known as Akt) predominantly regulate $p27^{Kip1}$ subcellular localization (26). Although the MAPK pathway is activated by ventilation/stretch (34-35) we found nuclear $p27^{Kip1}$ accumulation instead of degradation. Thus, MAPK may regulate $p27^{Kip1}$ differently in normal compared to cancer cells. The PI3K-Akt pathway during ventilation/stretch remains to be investigated. Mechanical ventilation of newborn rats triggers an inflammatory response(17-18) and various inflammatory mediators including tumor necrosis factor-a (TNFα), interleukin-6 and transforming growth factor-β (TGF-β) have been shown to induce $p21^{WAF1/Cip1}$ expression (22, 36-37). Also $p15^{Ink4b}$ is induced by TGF-β (38). In the current study, $TGF\beta_1$ mRNA expression was not changed after 24 h of ventilation (data not shown) and, indeed, neither $p21^{WAF1/Cip1}$ nor $p15^{Ink4b}$ expression was affected by mechanical ventilation.

The amount of $p27^{Kip1}$ is regulated at the level of its synthesis (transcription, translation), degradation and localization (39). During the G_0 phase, it accumulates in the nucleus and inhibits cyclin-Cdk complexes. In response to growth stimuli, $p27^{Kip1}$ translocates from the nucleus to the cytoplasm during G_1 phase and is degraded by the proteosome after ubiquitination (39), permitting the cell cycle to progress to S phase. Several signaling pathways that alter $p27^{Kip1}$ phosphorylation influence its subcellular localization and function. For example, phosphorylation of the following essential sites regulate important functions: Thr^{157} prevents nuclear import; Ser^{10} mediates nuclear export; Thr^{198} promotes cytoplasmic translocation and increases p27-dependent motility, which may be important to prepare cells for shape changes in later phases of the cell cycle; and, Thr^{187} results in proteolysis (26, 39). In the present study, mechanical ventilation of 24 h increased the transcription of $p27^{Kip1}$ and altered its phosphorylation: less phosphorylation of Thr^{157} (increasing nuclear import), Thr^{198} (decreasing nuclear export) and Thr^{187} (reduced proteolysis). No significant changes in Ser^{10} phosphorylation were noted (not shown). Together, these alterations in $p27^{Kip1}$ phosphorylation favour its nuclear localization and stability. In addition, the reduced phosphorylation of $p27^{Kip1}$ at Thr^{157} and Thr^{198} negatively affects the assembly function of $p27^{Kip1}$ for cyclinD1-Cdk4, thereby negatively affecting cell cycle progression (26).

The second family of CKIs consists of INK4 proteins ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$); they inhibit cyclin D-dependent kinases Cdk-4 and -6 (14-15) and, are thus specific for early G_1 phase. In the present study, we found a significant reduction in $p16^{INK4a}$ protein after ventilation with low, moderate or high V_T. In addition to Cdk inhibition and G_1 growth arrest, $p16^{INK4a}$ plays a role in regulating apoptosis. It has been shown that $p16^{INK4a}$-deficiency increases apoptosis in osteosarcoma U2OS and mouse embryonic fibroblast (MEF) cells exposed to ultraviolet (UV) light (40), because of down-regulation of the anti-apoptotic protein Bcl-2. In contrast, the pro-apoptotic protein Bax was down-regulated in $p16^{INK4a}$ expressing cells (40). Thus, $p16^{INK4a}$ appears to control apoptosis through the intrinsic mitochondrial death pathway. Prolonged

mechanical ventilation has been shown to significantly increase lung cell apoptosis in newborn mice lungs (8). Although $p16^{INK4a}$ levels were decreased in the present study, it remains yet to be determined whether it plays a role in ventilator-induced apoptosis.

Another risk factor for BPD is oxygen (1). Hyperoxia has been shown to interfere with cell-cycle progression *in vitro* (36, 41-42) and hyperoxia-induced G_1 arrest appears to be mediated by $p21^{Waf1/Cip1}$(43-44). The hyperoxic induction of $p21^{Waf1/Cip1}$ is p53-dependent (44) and its increase promotes survival of cells exposed to continuous oxidative stress by maintaining anti-apoptotic Bcl-2X(L) expression (45). Hyperoxic-ventilated premature baboons delivered at 125 and 140 days of gestation have increased p53 and $p21^{Waf1/Cip1}$ expression (46-47). In the present study, we did not assess p53 but the absence of $p21^{Waf1/Cip1}$ induction by 24 h of mechanical ventilation with room air suggests that p53 is likely not involved in ventilation-induced cell cycle arrest in these studies.

The increase of $p27^{Kip1}$ and $p57^{Kip2}$ by mechanical stretch *in vitro* and *in vivo* coincided with a reduced expression of cyclins D1 and E1, both of which are essential for cell cycle progression through G_1 and entry in S phase. D-type cyclins are induced by mitogenic stimuli in quiescent cells. After association with Cdk4/6 and activation by Cdk activating kinase, they promote entry into the G_1 phase, thereby triggering cyclin E expression. Cyclin E binds to Cdk2 and facilitates transition from G_1 to S phase (22). Both $p27^{Kip1}$ and $p57^{Kip2}$ are potent inhibitors of cyclin E-dependent kinase Cdk2, but at high concentrations they also block Cdks4/6. In addition, it is plausible that cell cycle progression is inhibited due to the reduced phosphorylation of $p27^{Kip1}$ at critical threonines (Thr157, Thr198) which negatively affects the assembly function of $p27^{Kip1}$ for cyclin D1-Cdk4 complexes (48). The down-regulation of cyclin D1 and E1 expression suggests a G_1 cell cycle arrest, a conclusion that is supported by the absence of BrdU incorporation (S-phase event) and positive PH3 staining (M-phase marker). In the 125-day premature born baboon model of BPD, the animals received ventilator support and oxygen as needed to achieve normal blood-gas values (49), and such treatment increased pulmonary expression of cyclin D1 and E at day 6 while prolonged ventilation and oxygen exposure led to a decrease in cyclin E (46). It is possible that lung cells were initially undergoing repair by increasing proliferation, but that prolonged exposure impairs the expression of cyclins, resulting in failure of repair and inhibition of further development. Furthermore, increased levels of the Cdk inhibitor $p21^{Waf1/Cip1}$ in the baboon BPD model (46) suggests that G_1 growth arrest may occur in infants with BPD. Unfortunately, the expression of $p27^{Kip1}$ or $p57^{Kip2}$ has not been investigated in the baboon BPD model.

In summary, we conclude that mechanical ventilation for 24 h using moderate V_T without supplemental O_2 causes G_1 cell cycle arrest of lung cells in newborn rats due to increased transcription and altered phosphorylation (in favour of nuclear localization)

of Kip CKIs, and down-regulation of cyclins D and E. This proliferative arrest may cause a reduction in alveolarization, resulting in alveolar simplification. Such identification of ventilation-induced CKIs may have therapeutic potential for the prevention -or treatment- of arrested alveolarization in ventilated premature infants

REFERENCES

1. Jobe AJ. The new BPD: an arrest of lung development. Pediatr Res. 1999 Dec;46(6):641-3.
2. Fanaroff AA, Stoll BJ, Wright LL, Carlo WA, Ehrenkranz RA, Stark AR, et al. Trends in neonatal morbidity and mortality for very low birthweight infants. Am J Obstet Gynecol. 2007 Feb;196(2):147 e1-8.
3. Husain AN, Siddiqui NH, Stocker JT. Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. Hum Pathol. 1998 Jul;29(7):710-7.
4. Cutz E, Chiasson D. Chronic lung disease after premature birth. N Engl J Med. 2008 Feb 14;358(7):743-5; author reply 5-6.
5. Kinsella JP, Greenough A, Abman SH. Bronchopulmonary dysplasia. Lancet. 2006 Apr 29; 367(9520):1421-31.
6. Baraldi E, Filippone M. Chronic lung disease after premature birth. N Engl J Med. 2007 Nov 8;357(19):1946-55.
7. Roth-Kleiner M, Post M. Similarities and dissimilarities of branching and septation during lung development. Pediatr Pulmonol. 2005 Aug;40(2):113-34.
8. Mokres LM, Parai K, Hilgendorff A, Ertsey R, Alvira CM, Rabinovitch M, et al. 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. Jan; 298(1):L23-35.
9. Sanchez-Esteban J, Wang Y, Cicchiello LA, Rubin LP. Cyclic mechanical stretch inhibits cell proliferation and induces apoptosis in fetal rat lung fibroblasts. Am J Physiol Lung Cell Mol Physiol. 2002 Mar;282(3):L448-56.
10. McAdams RM, Mustafa SB, Shenberger JS, Dixon PS, Henson BM, DiGeronimo RJ. Cyclic stretch attenuates effects of hyperoxia on cell proliferation and viability in human alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2006 Aug;291(2):L166-74.
11. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. 2003 Jun;36(3):131-49.
12. Park MT, Lee SJ. Cell cycle and cancer. J Biochem Mol Biol. 2003 Jan 31;36(1):60-5.
13. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. 1999 Jun 15;13(12):1501-12.
14. Parry D, Bates S, Mann DJ, Peters G. Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product. Embo J. 1995 Feb 1;14(3):503-11.
15. McConnell BB, Gregory FJ, Stott FJ, Hara E, Peters G. Induced expression of p16(INK4a) inhibits both CDK4- and CDK2-associated kinase activity by reassortment of cyclin-CDK-inhibitor complexes. Mol Cell Biol. 1999 Mar;19(3):1981-9.
16. Soos TJ, Kiyokawa H, Yan JS, Rubin MS, Giordano A, DeBlasio A, et al. Formation of p27-CDK complexes during the human mitotic cell cycle. Cell Growth Differ. 1996 Feb;7(2): 135-46.
17. Roth-Kleiner M, Ridsdale R, Cao L, Kuliszewski M, Tseu I, McKerlie C, et al. Lipopolysaccharide exposure modifies high tidal volume ventilation-induced proinflammatory mediator expression in newborn rat lungs. Pediatr Res. 2007 Feb;61(2):191-6.
18. Kroon AA, Wang J, Huang Z, Cao L, Kuliszewski M, Post M. Inflammatory response to oxygen and endotoxin in newborn rat lung ventilated with low tidal volume. Pediatr Res. 2010 Jul;68(1):63-9.

19. Yi M, Jankov RP, Belcastro R, Humes D, Copland I, Shek S, et al. Opposing effects of 60% oxygen and neutrophil influx on alveologenesis in the neonatal rat. Am J Respir Crit Care Med. 2004 Dec 1;170(11):1188-96.
20. Dunnill MS. Evaluation of a Simple Method of Sampling the Lung for Quantitative Histological Analysis. Thorax. 1964 Sep;19:443-8.
21. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976 May 7;72: 248-54.
22. Maddika S, Ande SR, Panigrahi S, Paranjothy T, Weglarczyk K, Zuse A, et al. Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy. Drug Resist Updat. 2007 Feb-Apr;10(1-2):13-29.
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.
24. Caniggia I, Tseu I, Han RN, Smith BT, Tanswell K, Post M. Spatial and temporal differences in fibroblast behavior in fetal rat lung. Am J Physiol. 1991 Dec;261(6 Pt 1):L424-33.
25. Copland IB, Reynaud D, Pace-Asciak C, Post M. Mechanotransduction of stretch-induced prostanoid release by fetal lung epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2006 Sep;291(3):L487-95.
26. Larrea MD, Wander SA, Slingerland JM. p27 as Jekyll and Hyde: regulation of cell cycle and cell motility. Cell Cycle. 2009 Nov 1;8(21):3455-61.
27. Burri PH. Lung development and pulmonary angiogenesis. In *Lung Development*, edited by Gaultier C, Bourbon J, Post M. New York, Oxford University Press, 1999, 122-11.
28. Kauffman SL, Burri PH, Weibel ER. The postnatal growth of the rat lung. II. Autoradiography. Anat Rec. 1974 Sep;180(1):63-76.
29. Susaki E, Nakayama KI. Functional similarities and uniqueness of p27 and p57: insight from a knock-in mouse model. Cell Cycle. 2009 Aug 15;8(16):2497-501.
30. De Paepe ME, Johnson BD, Papadakis K, Luks FI. Lung growth response after tracheal occlusion in fetal rabbits is gestational age-dependent. Am J Respir Cell Mol Biol. 1999 Jul; 21(1):65-76.
31. Nardo L, Maritz G, Harding R, Hooper SB. Changes in lung structure and cellular division induced by tracheal obstruction in fetal sheep. Exp Lung Res. 2000 Mar;26(2):105-19.
32. Xu J, Liu M, Tanswell AK, Post M. Mesenchymal determination of mechanical strain-induced fetal lung cell proliferation. Am J Physiol. 1998 Sep;275(3 Pt 1):L545-50.
33. Edwards YS, Sutherland LM, Power JH, Nicholas TE, Murray AW. Cyclic stretch induces both apoptosis and secretion in rat alveolar type II cells. FEBS Lett. 1999 Apr 1;448(1): 127-30.
34. Ngiam N, Peltekova V, Engelberts D, Otulakowski G, Post M, Kavanagh BP. Early growth response-1 worsens ventilator-induced lung injury by up-regulating prostanoid synthesis. Am J Respir Crit Care Med. 2010 May 1;181(9):947-56.
35. Copland IB, Post M. Stretch-activated signaling pathways responsible for early response gene expression in fetal lung epithelial cells. J Cell Physiol. 2007 Jan;210(1):133-43.
36. Corroyer S, Maitre B, Cazals V, Clement A. Altered regulation of G1 cyclins in oxidant-induced growth arrest of lung alveolar epithelial cells. Accumulation of inactive cyclin E-DCK2 complexes. J Biol Chem. 1996 Oct 11;271(41):25117-25.

37. Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, et al. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell. 1994 Jul 15;78(1):59-66.
38. Hannon GJ, Beach D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature. 1994 Sep 15;371(6494):257-61.
39. Susaki E, Nakayama KI. Multiple mechanisms for p27(Kip1) translocation and degradation. Cell Cycle. 2007 Dec 15;6(24):3015-20.
40. Al-Mohanna MA, Manogaran PS, Al-Mukhalafi Z, K AA-H, Aboussekhra A. The tumor suppressor p16(INK4a) gene is a regulator of apoptosis induced by ultraviolet light and cisplatin. Oncogene. 2004 Jan 8;23(1):201-12.
41. McGrath SA. Induction of p21WAF/CIP1 during hyperoxia. Am J Respir Cell Mol Biol. 1998 Feb;18(2):179-87.
42. Clement A, Henrion-Caude A, Besnard V, Corroyer S. Role of cyclins in epithelial response to oxidants. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 2):S81-4.
43. Rancourt RC, Keng PC, Helt CE, O'Reilly MA. The role of p21(CIP1/WAF1) in growth of epithelial cells exposed to hyperoxia. Am J Physiol Lung Cell Mol Physiol. 2001 Apr; 280(4):L617-26.
44. Helt CE, Staversky RJ, Lee YJ, Bambara RA, Keng PC, O'Reilly MA. The Cdk and PCNA domains on p21Cip1 both function to inhibit G1/S progression during hyperoxia. Am J Physiol Lung Cell Mol Physiol. 2004 Mar;286(3):L506-13.
45. Staversky RJ, Vitiello PF, Gehen SC, Helt CE, Rahman A, Keng PC, et al. p21(Cip1/Waf1/Sdi1) protects against hyperoxia by maintaining expression of Bcl-X(L). Free Radic Biol Med. 2006 Aug 15;41(4):601-9.
46. Das KC, Ravi D. Altered expression of cyclins and cdks in premature infant baboon model of bronchopulmonary dysplasia. Antioxid Redox Signal. 2004 Feb;6(1):117-27.
47. Maniscalco WM, Watkins RH, Roper JM, Staversky R, O'Reilly MA. Hyperoxic ventilated premature baboons have increased p53, oxidant DNA damage and decreased VEGF expression. Pediatr Res. 2005 Sep;58(3):549-56.
48. Larrea MD, Liang J, Da Silva T, Hong F, Shao SH, Han K, et al. Phosphorylation of p27Kip1 regulates assembly and activation of cyclin D1-Cdk4. Mol Cell Biol. 2008 Oct;28(20): 6462-72.
49. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. Am J Respir Crit Care Med. 1999 Oct;160(4):1333-46.

Chapter 7

Alterations in Expression of Elastogenic and Angiogenic Genes by Mechanical Ventilation in Newborn Rat Lung

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Submitted

ABSTRACT

Mechanical ventilation, inflammation and hyperoxia are important risk factors for the development of bronchopulmonary dysplasia. Here we investigated the effects of different tidal volumes (V_T), inflammation, hyperoxia and duration of ventilation on the expression of genes involved in alveolarization (tropoelastin (TE), lysyl oxidase (LOX), fibulin5 (Fib5) and tenascin-C (TN-C) and angiogenesis (platelet derived growth factors (PDGF) and vascular endothelial growth factors (VEGF)) in newborn rats. First, eight-day old rats were ventilated for 8 hours (h) with low (LV_T: 3.5 ml.kg^{-1}), moderate (MV_T: 8.5 ml.kg^{-1}) or high (HV_T: 25 ml.kg-1) tidal volumes. LV_T and MV_T ventilation for 8 h hardly influenced the expression of TE, LOX and Fib5, while TN-C expression was significantly decreased. HV_T ventilation increased gene expression of all three elastogenic genes and TN-C. PDGF-α receptor (PDGF-Rα) mRNA, but not protein, was significantly increased in all ventilation groups. PDGF-A gene expression was not affected by ventilation, but PDGF-B mRNA levels were decreased after MV_T and HV_T ventilation. VEGF expression was only up-regulated after HV_T ventilation, while, independent of V_T, ventilation up-regulated VEGF receptor 1 (VEGF-R1) expression. In contrast, VEGF-R2 expression was down-regulated by MV_T and HV_T ventilation. Next we studied the effect of pre-exposure to LPS together with LV_T ventilation with room air and 50% oxygen on expression on these genes. Pre-exposure to LPS decreased the expression of Fib5, while oxygen increased Fib5 mRNA expression, which was abolished by pre-exposure to LPS. Pre-exposure to LPS and LV_T-ventilation with oxygen did not influence the expression of the angiogenic genes. Finally, we evaluated the duration (0-24 hours) of MV_T ventilation. Although expression of all three elastogenic genes peaked at 12 hours of ventilation, only that of Fib5 was negatively affected at 24 hours. In contrast, TN-C expression decreased with duration of ventilation. Disturbed elastin fiber deposition was only noticeable after 24 hours of ventilation. Ventilation-induced changes in expression of PDGF and VEGF receptors were maximal at 8 hours of ventilation, but a morphometric decrease in small vessel density was only observed after 24 hours. Thus, an imbalance between Fib5 and TE expression may trigger dys-regulated elastin fiber deposition during the first 24 h of mechanical ventilation. This imbalance in expression is accelerated by a systemic inflammation. Furthermore, ventilation-induced alterations in PDGF and VEGF receptors expression are tidal volume dependent and affect pulmonary vessel formation. Taken together the data are compatible with mechanical ventilation disturbing normal elastogenesis and angiogenesis in newborn rat lungs.

INTRODUCTION

Bronchopulmonary dysplasia (BPD), a chronic lung disease of premature born infants, is a major cause of morbidity and mortality. In severely affected infants symptoms last into early adulthood (1, 2). BPD is a consequence of an inflammatory lung injury that leads to remodeling of the lung. Inflammation results from a variety of stimuli, of which mechanical ventilation, hyperoxia and infection are most prominent. The incidence of BPD increases with decreasing gestational age. Due to the improved survival of extreme premature infants, the pathological features of BPD have evolved since its first description in 1967 by Northway *et al*. (3). Nowadays the pathology of BPD is characterized by alveolar symplification and enlargement, and dysmorphic vasculature. It is not surprising that these findings are believed to be the result of an arrest in alveolar development. After all, lungs of infants born at 24-32 weeks are in the saccullar stage of development and structurally and biochemically immature. During this period of development extensive vasculogenesis takes place within the developing terminal sacculi, followed by secondary crests formation with interstitial extracellular matrix loss and remodelling with epithelial morphogenesis and capillary growth (4). Among the diverse factors that contribute to normal lung development, elastin, platelet-derived growth factors (PDGF) and vascular endothelial growth factor (VEGF) play a prominent role (5). However, the effect of each BPD risk factor, alone or combined on these regulatory molecules of alveolar and vascular development is still not understood.

Lung pathology of children with BPD revealed irregularly distributed alveolar elastic fibers (6). Other studies have shown increases in hydroxyproline (reflecting collagen) and elastin in lung injuries after ventilation (7, 8). Chronically ventilated preterm lambs have abundant and disordered elastin fibers (9) and display increased expression of tropoelastin (TE) and lysyl oxidase-like-1 (LOXL1): molecules involved in the assembly of elastin fibers. A recent study showed that blocking lung elastase in newborn mice ventilated with 40% oxygen prevented elastin degradation and preserved lung growth (10), providing evidence that ventilatory (stretch)-induced changes in lung elastin homeostasis impairs alveolar development.

The PDGF signalling network consists of four isoforms PDGFA-D, and two receptors, PDGF-Rα and PDGF-Rβ. PDGF-A is needed for the development of alveolar myofibroblasts, which produce elastin. PDGF-B has been linked to compensatory lung growth and pulmonary vascular remodeling (11, 12). Vascular endothelial growth factor (VEGF) is a specific endothelial cell mitogen that regulates endothelial cell differentiation, angiogenesis, and maintenance of existing vessels (13). Mechanical ventilation has been shown to decrease pulmonary expression for VEGF, PDGF-A, and their receptors (VEGF-R2, PDGF-Rα) in preterm and term lambs with BPD (9), thereby likely impacting pulmonary vascular development.

The main objective of this study was to investigate the effects of mechanical ventilation on pulmonary expression of genes that regulate formation of alveoli and blood vessels in lungs of newborn rats, in which alveolarization and angiogenesis occur mainly after birth (14). We hypothesized that the expression of these genes would be stretch-amplitude and time dependent. Furthermore, oxygen and a systemic inflammation would further alter the expression of these genes. We observed that fibulin5 and PDGF-B are the first genes to be down-regulated by moderate tidal volume ventilation. Inflammation accelerates this negative effect of mechanical ventilation on fibulin5 expression, while low tidal volume ventilation delays this downregulatory effect of moderate mechanical ventilation on the expression of PDGF-B.

METHODS

Ethics statement: The study was conducted according to the guidelines of the Canadian Council for Animal Care and with approval of the Animal Care Review Committee of the Hospital for Sick Children (protocol #7217).

Animal preparation: Timed pregnant Wistar rats (Charles River, Oakville, Quebec, Canada) were allowed to deliver and immediately afterwards litters were reduced to 10 pups. In three series of experiments newborn rats were ventilated to assess the effect of different tidal volumes, inflammation, oxygen and duration of ventilation on alveolar development. They were anesthetized by *i.p.* injection of 30 mg kg^{-1} pentobarbital and a tracheotomy was performed. The trachea was cannulated with a 1 cm 19G cannula and connected to a rodent ventilator (FlexiVent Scireq, Montreal, PQ). Isoflurane was used as general anesthesia and 0.9% saline (100 $ml.kg^{-1}$/24h) was administered subcutaneously by continuous infusion with a 27G needle to prevent dehydration. Preliminary experiments were performed to determine ventilator settings (15). Animals were monitored by ECG. Rectal temperature was maintained at 37°C using a thermal blanket, lamp and plastic wrap. At the end of the ventilation period a blood sample from the carotid artery was taken for blood gas analysis prior to euthanasia. Lung tissues were processed for histology or flash frozen for molecular/protein analyses.

Mechanical ventilation:

Series I: Different Tidal Volumes. Rat pups (postnatal day 8) were randomly assigned to one of the following four groups: 1) Non-ventilated (NV) controls; 2) Low V_T (V_T 3.5 $ml.kg^{-1}$, freq. 600 $minute^{-1}$, PEEP 0 cm H_2O; increasing PEEP to 2 cm H_2O led to an increase of pCO_2 and early death); 3) Moderate V_T (8.5 $ml.kg^{-1}$, RR 150 min^{-1}, PEEP 2

cm H_2O); and 4) High V_T (V_T 25 ml.kg^{-1}, freq. 20 minute^{-1}, PEEP 2 cm H2O). Pups were ventilated for 8 hrs.

Series II: Pre-exposure to LPS and Low V_T Ventilation with Oxygen. Rat pups (postnatal day 8) were randomly assigned to injection (*ip*) of either 3 mg.kg^{-1} body weight of LPS from *E. coli* serotype 026:B6 or the same volume of 0.9% NaCl (14). Twenty four hours after treatment animals were randomly assigned to one of the following six groups: 1) Non-ventilated after NaCl injection (NV); 2) NV after LPS injection (NV+LPS); 3) Low V_T (V_T 6 ml.kg^{-1}, freq. 600 minute^{-1}, PEEP 0 cm H_2O) with room air after NaCl injection (LV_T): 4) Low V_T with room air after LPS injection (LV_T + LPS); 5) Low V_T with 50% oxygen after NaCl injection (LV_T+O_2); 6) Low V_T with 50% oxygen after LPS injection (LV_T+LPS/O_2). Pups were ventilated for 8 hrs.

Series III: Duration of Ventilation. Rat pups (postnatal days 7) were ventilated with room air and moderate-V_T (8.5 mL.kg^{-1}, RR 150 min^{-1}, PEEP 2 cm H_2O) for 12 and 24 h.

Histology: After flushing whole lungs were infused in *situ* with 4% (w/v) paraformaldehyde (PFA) in PBS with a constant pressure of 20 cm H_2O over 5 minutes to have equalized filling pressure over the entire lung. Under these constant pressure conditions the cannula was removed and the trachea immediately ligated. The lungs were excised and immersed in 4% PFA in PBS overnight, dehydrated and embedded in paraffin. Sections of 5 µm were stained for elastin using accustain artrazine solution (Sigma, St. Louis MO, USA).

Immunohistochemistry: Following sectioning and antigen retrieval by heating in 10 mM sodium citrate pH 6.0, sections were washed in PBS and endogenous peroxidase was blocked in 3% (v/v) H_2O_2 in methanol. Blocking was done with 5% (w/v) normal goat serum (NGS) and 1% (w/v) bovine serum albumin (BSA) in PBS. Sections were then incubated overnight at 4°C with 1:500 diluted rabbit anti-Tenascin (Chemicon Inc, Huissen, Netherlands). Biotinylated goat anti-rabbit IgG (1:2000) was used as secondary antibody. Color detection was performed according to instruction in the Vectastain ABC and DAB kit (Vector Laboratories, Burlingname, CA, USA). All sections were counterstained with hematoxylin.

Quantitative RT-PCR: Lung tissues were homogenized in Trizol® (Invitrogen Canada Inc, Burlington, ON, Canada) and total RNA was extracted according to the manufacturer's protocol. Total RNA was treated with DNAse I® (Invitrogen Canada Inc, Burlington, ON, Canada) at 37°C to remove residual genomic DNA. Total RNA was reverse transcribed using Superscript II reverse transcriptase® (Invitrogen Canada Inc,

Table 1. Primer sequences for SYBR Green real-time PCR

Gene	Primer	Sequence
VEGFR1	Forward	GAGTGTGAACGGCTGCCCTATGATG
	Reverse	GATGGCCGATGTGGGTCAAG
PDGFA	Forward	CATTTGGCTGGGAAGACGGA
	Reverse	CCCACAGGCCAGCTTACTTATT
PDGFB	Forward	TCTCTGCTGCTACCTGCGTCTG
	Reverse	GAGCTTTCCGACTCGACTCCA
PDGF-Rα	Forward	GGTGACCTGCGCCGTCTTTA
	Reverse	GGCGGGCAGCACATTCATAATC
PDGF-Rβ	Forward	TGGCCTTGGTGGTCCTTACTGT
	Reverse	CTCCACCACCTGTCCGAAAGC

VEGFR: vascular endothelial growth factor receptor, PDGF: platelet-derived growth factor, PDGR-R: platelet-derived growth factor receptor.

Burlington, ON, Canada) and random hexamers (Applied Biosystems, Foster City, CA, USA). Complementary DNA was quantified in a 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Amplification was performed with AmpliTAq Gold polymerase (Applied Biosystems, Foster City, ON, Canada) using TaqMan primers and probes (Applied Biosystems, Foster City, ON, Canada) for the target genes T1-α, tropoelastin (TE) and TN-C (17). VEGF, VEGFR2, PECAM, lysyl oxidase (LOX), fibulin 5 and 18S were Assays on Demand® from Applied Biosystems (Foster City, ON, Canada). VEGFR1, PDGF-A, PDGF-B, PDGF-Rα and PDGF-Rβ cDNA (see Table 1) were quantified using SYBR Green. For relative quantification, polymerase chain reaction signals were compared between groups after normalization using 18S as an internal reference. Fold change was calculated according to Livak and Schmittgen (16).

Vessel numbers: The number of peripheral vessels of <20 μm and 20–65 μm in diameter were counted in 30 random, non-overlapping fields per lung with all fields being within 435 μm of the edge of the lung on tissue cross-section as previously described (17).

Statistical analysis: Stated otherwise all data are presented as mean ± SD. Data was analyzed using SPSS software version 15 (SPSS Inc, Chicago, IL). Statistical significance ($p < 0.05$) was determined by using one-way ANOVA or Kruskal-Wallis test. *Post hoc* analysis was performed using Duncan's multiple-range test (data presented as mean ± SD) or Mann-Whitney test (data presented as median and interquartile range).

RESULTS

Physiologic data: *Series 1:* Blood gases were in the normal range after eight hours of ventilation with different tidal volumes (Table 2A) (15). *Series 2:* Ventilation for 8 hours with low V_T with room air after exposure to LPS (LV_T+LPS), ventilation with 50% oxygen (LV_T+O_2), and ventilation with oxygen after exposure to LPS (LV_T+LPS/O_2) resulted also in normal pH and $PaCO_2$ (Table 2B). Low V_T *v*entilation with room air after exposure to

Table 2A. Airway pressures and blood gas analysis after 8 hours of mechanical ventilation with low, moderate and high V_T (Series 1).

	Groups		
	LV_T	MV_T	HV_T
pH	7.39 ± 0.07	7.39 ± 0.03	7.39 ± 0.04
pCO_2 (mmHg)	45 ± 4.4	44 ± 6.4	42 ± 2.2
pO_2 (mmHg)	72 ± 14.0	83 ± 8.4	91 ± 9.8
Ppeak (cm H_2O)	6.0 ± 0.5	10.1 ± 1.5*	19.9 ± 0.4**
Pmean (cm H_2O)	3.6 ± 0.26	6.4 ± 1.0*	10.8 ± 0.6**
PEEP (cm H_2O)	0	2	2
Delivered V_T (ml/kg)	~3.5	~8.5	~25
Frequency (breaths min^{-1})	600	160	20

Statistical analysis (ANOVA) was done for measured values only. Values represent means ± SD, n = 4 animals per group. *p < 0.05 *vs*. animals ventilated with LV_T and HV_T; ** p <0.05 *vs*. animals ventilated with LV_T and MV_T. LV_T, low tidal volume; MV_T, moderate tidal volume; HV_T, high tidal volume; Pmean, mean pressure; Ppeak, peak pressure; PEEP, positive-end expiratory pressure.

Table 2B. Airway pressures and blood gas analysis after 8 hours of low V_T mechanical ventilation with room air or 50% oxygen and after exposure to LPS (Series 2)

	Groups			
	LV_T	LV_T+LPS	LV_T+O_2	LV_T+LPS/O_2
pH	7.36 ± 0.16	7.40 ± 0.04	7.32 ± 0.2	7.30 ± 0.08
pCO_2 (mmHg)	40.7 ± 6.6	36.0 ± 3.9	58.3 ± 28.8	54.3 ± 16.6
pO_2 (mmHg)	67.2 ± 10.4	87.7 ± 6.7*	131 ± 42.5*	167 ± 40.4**
Pmean (cm H_2O)	4.2 ± 0.6	4.2 ± 0.4	4.6 ± 0.6	4.3 ± 0.8
Pmax (cm H_2O)	6.6 ± 0.6	6.7 ± 0.4	7.4 ± 0.6	6.8 ± 1.2
PEEP (cm H_2O)	0	0	0	0
Delivered V_T (ml/kg)	~3.5	~3.5	~3.5	~3.5
Frequency (breaths min^{-1})	600	600	600	600

Statistical analysis (ANOVA) was done for measured values only. Group LV_T+LPS and group LV_T+O_2 were not compared. Values represent mean ± SD, n = 4 animals per group. LV_T, ventilated with low tidal volume; LV_T+LPS, ventilated with LV_T after exposure to LPS; LV_T+O_2, ventilated with low LV_T and 50% oxygen; LV_T+LPS/O_2, ventilated with LV_T and 50% oxygen after exposure to LPS. LV_T+O_2 group was not compared with LV_T+LPS. *p < 0.05 *vs*. animals ventilated with LV_T; **p < 0.05 *vs*. animals ventilated with LV_T and LV_T+LPS.

Table 2C. Blood gas analysis and airway pressures after 8, 12 and 24 hours of mechanical ventilation with room air (Series 3).

	Groups		
	8 hours	12 hours	24 hours
pH	7.39 ± 0.07	7.29 ± 0.05	7.30 ± 0.05
pCO_2 (mmHg)	44.3 ± 6.4	35.8 ± 6.2	39.4 ± 4.5
pO_2 (mmHg)	83 ± 8.4	87.4 ± 11.2	73.5 ± 11.7
BE (mmol/L)	-4.2 ± 2.3	-3.9 ± 1.6	-5.2 ± 2.1
Ppeak (cm H_2O)	10.9 ± 1.1	12.7 ± 1.1*	13.1 ± 1.3*
Pmean (cm H_2O)	6.3 ± 0.5	7.2 ± 0.5*	7.5 ± 0.6*
PEEP (cm H_2O)	2	2	2
Delivered V_T (ml/kg)	8.9 ± 0.2	8.5 ± 0.4*	8.5 ± 0.2*
Frequency	150	150	150

Statistical analysis (ANOVA) was done for measured values only Values represent mean ± SD, n = 12 animals per group. *$p < 0.05$ versus values at 0 hrs. Ppeak, peak pressure; Pmean, mean pressure; PEEP, positive-end expiratory pressure.

LPS (LV_T +LPS) and ventilation with 50% oxygen (LV_T+O_2) significantly increased the PaO_2 when compared to ventilation with room air (LV_T). The combination of ventilation with oxygen and exposure to LPS (LV_T+LPS/O_2) further increased PaO_2 *vs*. LV_T and LV_T+LPS groups ($p < 0.05$). Mean airway pressures and peak pressures remained stable during the ventilation period and were not different between groups in series 1 and 2 (Table 2A, 2B). *Series 3:* Again, blood gases were in the normal range after 8, 12 and 24 h of ventilation (Table 2C) (18). Mean airway pressures, peak pressures and delivered V_T remained constant up to 8 h of ventilation, but altered slightly after 12 h of ventilation compared to baseline (Table 2C).

Expression of matrix molecules implicated in alveolarization: *Series 1:* Eight hours of ventilation with ~3.5 ml. kg^{-1} and ~8.5 $ml.kg^{-1}$ decreased the expression of tenascin-C (TN-C) *versus* non-ventilated control animals (Fig. 1A). In contrast, injurious ventilation with ~25 $ml.kg^{-1}$ increased the expression of TN-C. The expression of TN-C between LV_T and MV_T group was not significantly different. Immunohistochemical analysis showed that the TN-C distribution pattern was similar in non-ventilated and ventilated lungs with different tidal volumes (Fig. 2. Lower panels, HV_T *vs*. non-ventilated group). TN-C staining was mesenchymal and no immunoreactivity was found in the larger airways and blood vessels. Western blotting did not reveal any differences in TN-C protein expression (data not shown). Gene expression of lysyl oxidase (LOX) and tropoelastin (TE) was increased versus non-ventilated group after 8 h of ventilation with ~3.5 ml. kg^{-1}. Fibulin 5 (Fib5) was not affected by low and MV_T ventilation, while it was increased by injurious ventilation with ~25 $ml.kg^{-1}$ (Fig. 1A). TE, LOX and Fib5 regulate elastin synthesis and assembly, a process crucial for alveologenesis. Although gene expression

of these elastogenic genes was increased after injurious HV_T ventilation, a similar pattern of elastin deposition and arrangement was observed in lungs of all groups (Fig 2. Upper levels, HV_T *vs.* non-ventilated group). Expression of type I cell marker (T1-α) was only increased by injurious ventilation with ~25ml.kg^{-1}(Fig. 1A). *Series 2:* Exposure to LPS decreased the expression of TN-C *versus* non-ventilated control animals (Fig. 1B).

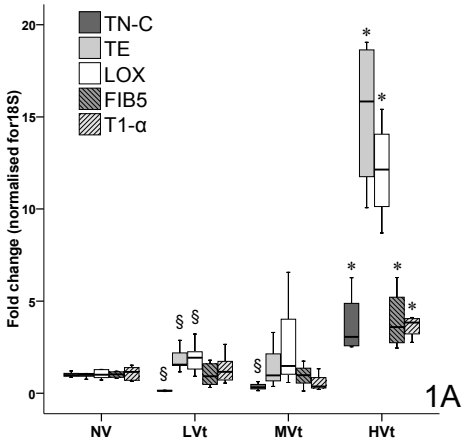

Figure 1A. Effect of low (LV_T), moderate (MV_T), and high (HV_T) ventilation for 8 hours with room air on the expression of matrix genes matrix genes implicated in alveolarization in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. *p < 0.05 *vs.* other groups; §p < 0.05 *vs.* NV.

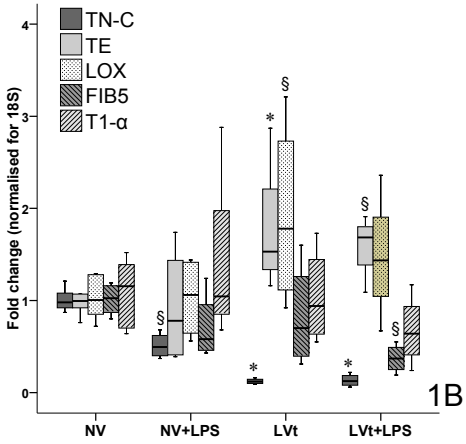

Figure 1B. Effect of exposure to LPS, low V_T ventilation (8 hours) with room air and low V_T ventilation (8 hours) after exposure to LPS on the expression of matrix genes implicated in alveolarization in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV: non ventilated (n = 8); NV+LPS: exposure to LPS, non ventilated (n = 5); LV_T: ventilated with low tidal volume (n = 6); LV_T+LPS; ventilated with LV_T after exposure to LPS (n = 5). §p < 0.05 *vs.* NV; *p < 0.05 *vs.* NV, NV+LPS.

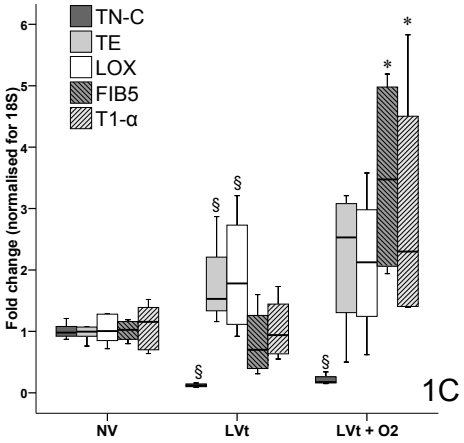

Figure 1C. Effect of low V_T ventilation (8 hours) with room air and low V_T ventilation (8 hours) with 50% oxygen on the expression of matrix genes implicated in alveolarization in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV: non ventilated (n=8); LV_T: ventilated with low tidal volume (n=6); LV_T+ O_2: ventilated with LV_T with 50% oxygen (n=5). §p < 0.05 *vs.* NV; *p < 0.05 *vs.* other groups.

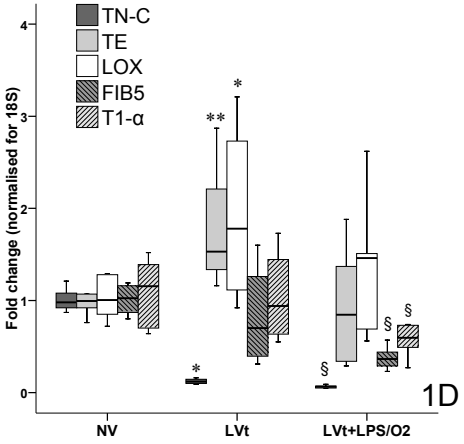

Figure 1D. Effect of exposure to low V_T ventilation (8 hours) with room air and low V_T ventilation (8 hours) with 50% oxygen after exposure to LPS on the expression of matrix genes implicated in alveolarization in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV: non ventilated (n = 8); LV_T: ventilated with low tidal volume (n=6); LV_T+LPS/O_2: ventilated with LV_T with 50% oxygen after exposure to LPS (n=6). *p < 0.05 *vs.* NV; **p < 0.05 *vs.* NV, LV_T+LPS/O_2; §p < 0.05 *vs.* other groups.

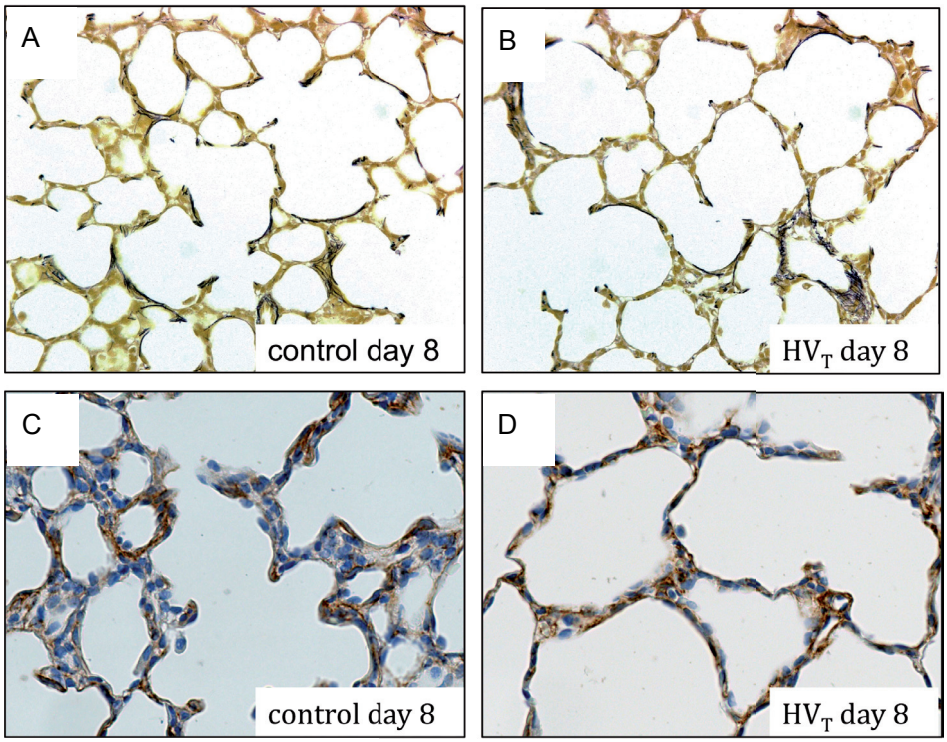

Figure 2. Immunostaining for elastin (Hart's staining) (upper panels) and immunostaining for TN-C (lower panels).

Ventilation with LV_T after exposure to LPS did further decrease the expression of TN-C *versus* non-ventilated control animals exposed to LPS but not *versus* LV_T ventilated animals. Also did LV_T ventilation after exposure to LPS decrease the gene expression of Fib5 and increase the gene expression of TE *versus* non-ventilated control animals (Fig. 1B). LV_T ventilation with 50% oxygen increased the gene expression of Fib5 and T1-α *versus* non-ventilated and LV_T ventilated animals (Fig. 1C). The combination of LV_T ventilation with 50% oxygen after exposure to LPS decreased the expression of TN-C, Fib5 and T1-α (Fig. 1D). Immunohistochemical analysis showed no differences in TN-C distribution pattern between different groups (data not shown). *Series 3:* A 24h-ventilation with moderate V_T further decreased the gene expression of TN-C *versus* 8 and 12 h of MV_T ventilation (Fig. 3A) without changing TN-C immunolocalization. Interestingly a peak of gene expression of LOX, TE and Fib5 was found after 12 h of MV_T ventilation (Figs. 3B, C, D). Although 24 h of MV_T ventilation did increase the expression of these genes *versus* non-ventilated animals, it was reduced compared to 12 h of MV_T ventilation (Figs. 3C, D). After 24 h of MV_T ventilation a different pattern of elastin deposition was observed. In non-ventilated animals and animals ventilated for 8 h, elastin was expressed mainly at the tips of the alveolar septa, whereas after 24 h of

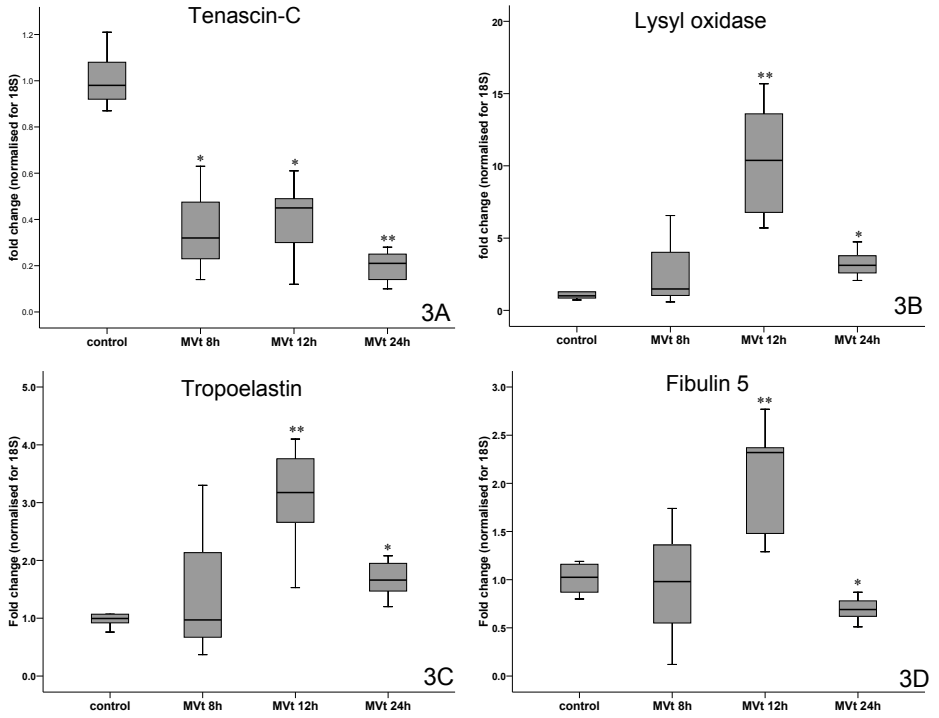

Figure 3. Effect of prolonged (8-24 hours) moderate (MV_T) ventilation with room air on the expression of matrix genes implicated in alveolarisation in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. Nonventilated controls (n=8), 8h MV_T (n=8), 12h MV_T (n=4) and 24 h MVT (n=4). **A**) Tenascin C, *p <0.05 *vs*. NV, **p < 0.05 *vs*. other groups; **B**) Lysyloxidase, *p < 0.05 *vs*. NV, **p < 0.05 *vs*. NV, 24 hours; **C**) Tropoelastin, *p < 0.05 *vs*. NV, **p < 0.05 *vs*. NV, 24 hours; **D**) Fibulin 5, *p < 0.05 *vs*. NV, **p < 0.05 *vs*. NV, 24 hours.

MV_T ventilation elastic fibers were prominent throughout the walls of distal respiratory units in the lungs (Fig. 4). The gene expression of T1-α was not altered after 24 h of MV_T ventilation (Fig. 5A).

Increased lung MMP-9 activity is associated with the development of BPD in newborn infants (19, 20) as well as in animal models (21, 22). We observed an increase in gene expression of MMP-9 after 12 h of MV_T ventilation, but not after 24 h of MV_T ventilation (Fig. 5B).

Expression of angiogenic molecules implicated in alveolarization: *Series 1:* Transcript levels of PDGF-Rα and PDGF-Rβ were significantly increased in all ventilation groups *versus* non-ventilated animals (Fig. 6). The increase in expression of PDGF-Rα was much more pronounced. No changes in PDGF-Rα immunoreactivity were detected (not shown). While PDGF-A expression was not altered, the number of PDGF-B transcripts was significantly decreased after MV_T and HV_T ventilation *versus* non- and LV_T

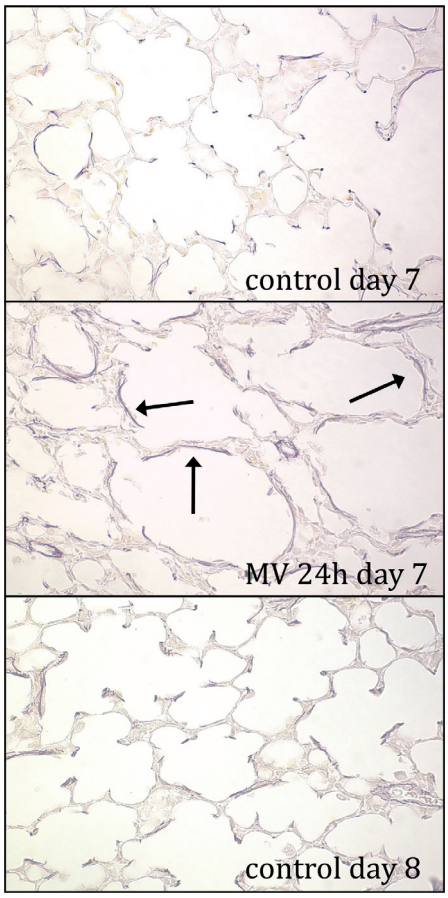

Figure 4. Immunostaining for elastin (Hart's staining): 7 day-old rat non-ventilated upper panel; 7 day-old rat ventilated for 24 h middle panel and 8 day-old rat non-ventilated (lower panel).

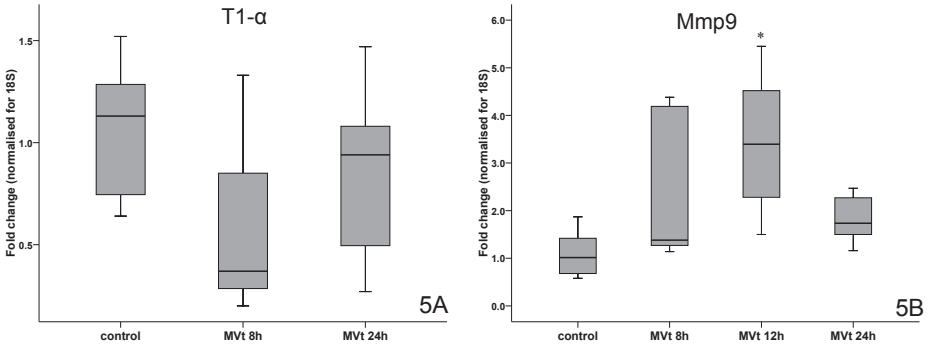

Figure 5. Effect of prolonged (8-24 hours) moderate (MV_T) ventilation with room air on the expression of T1-α (type I cell marker) and matrix metallopeptidase 9 (MMP9) in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. Nonventilated controls (n=8), 8h MV_T (n=8), and 24 h MVT (n=4). **A**) T1-a; **B**) MMP9, *$p < 0.05$ *vs.* NV, 24 hours.

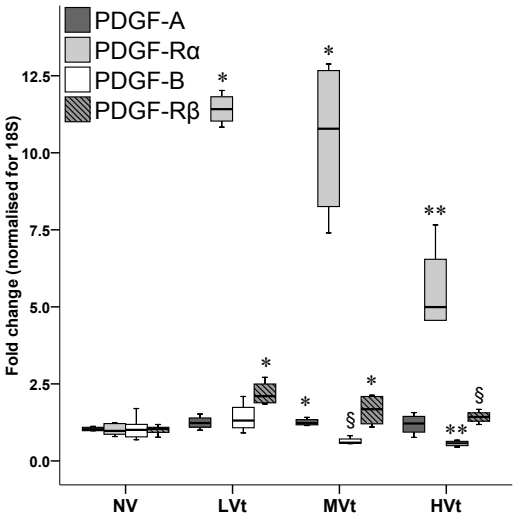

Figure 6. Effect of low (LV_T), moderate (MV_T) and high (HV_T) ventilation for 8 hours with room air on the expression of PDGF genes in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are the 5th and 95th percentiles. *p < 0.05 *vs.* NV, §p < 0.05 *vs.* NV, LV_T **p < 0.05 *vs.* other groups.

ventilation (Fig. 6). *Series 3:* Although 24 h MV_T ventilation also increased PDGF-Rα expression, this increase was less compared to 8 h MV_T ventilation (Fig 7A). The altered expression of PDGF-B and PDGF-Rβ after 8 h MV_T ventilation was normalized after 24 h MV_T ventilation (Fig 7B).

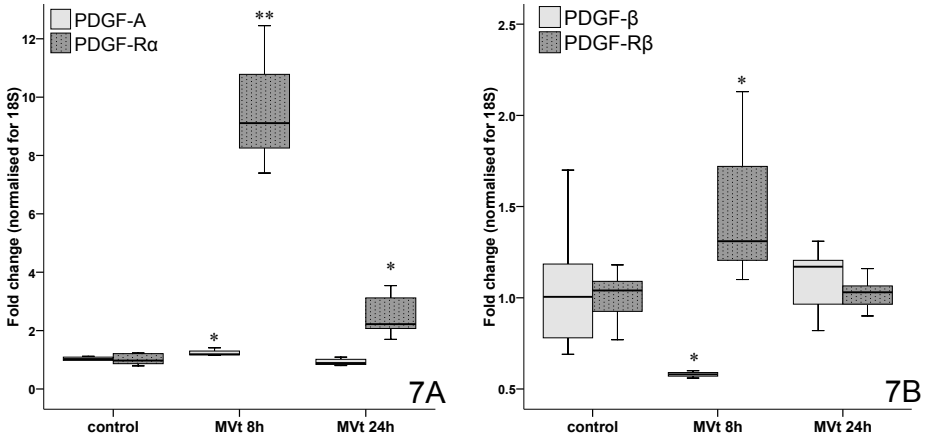

Figure 7. Effect of 8 and 24 hours of moderate (MV_T), ventilation with room air on the expression of PDGF genes in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. **A)** PDGF-A signalling, *P<0.05 *vs.* NV, **p < 0.05 *vs.* NV, 24hours; **B)** PDGF-B sigalling, *p < 0.05 *vs.* NV, 24 hours.

Various studies have shown the importance of vascular growth for lung development. VEGF is a potent inducer of endothelial cell growth that acts on vascular endothelial cells through two different receptors, VEGF-R1 and VEGF-R2. Inhibition of VEGF signaling has been shown to impair postnatal rat alveolar development (23). *Series 1:* VEGF-R1 expression was significantly up-regulated after LV_T, MV_T and HV_T ventilation, whereas VEGF-R2 expression was down-regulated by tidal volumes of ~8.5 $ml.kg^{-1}$ *vs*. other

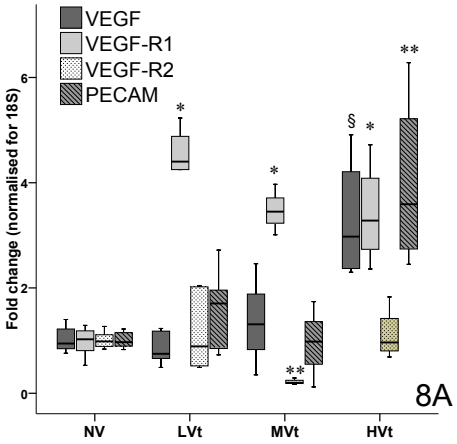

Figure 8A. Effect of low (LV_T), moderate (MV_T), and high (HV_T) ventilation for 8 hours with room air on the expression of matrix genes controlling vascularisation in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. *$p < 0.05$ *vs*. NV; §$p < 0.05$ *vs*. NV, LV_T; **$p < 0.05$ *vs*. other groups.

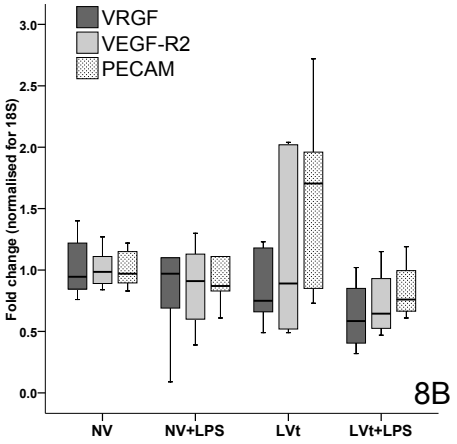

Figure 8B. Effect of exposure to LPS, low V_T ventilation (8 hours) with room air and low V_T ventilation (8 hours) after exposure to LPS on the expression of matrix genes controlling vascularisation in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV: non ventilated (n = 8); NV+LPS: exposure to LPS, non ventilated (n = 5); LV_T: ventilated with low tidal volume (n = 6); LV_T+LPS; ventilated with LV_T after exposure to LPS (n = 5).

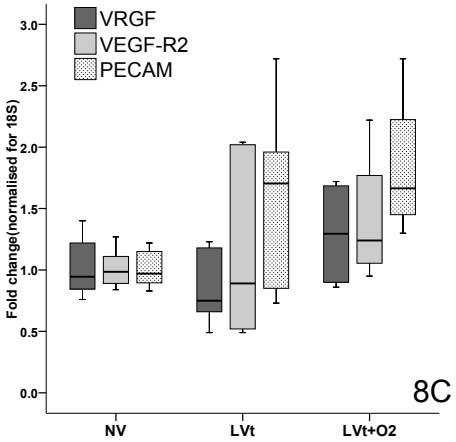

Figure 8C. Effect of low V_T ventilation (8 hours) with room air, low V_T ventilation (8 hours) with 50% oxygen on the expression of matrix genes controlling vascularisation in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV: non ventilated (n=8); LV_T: ventilated with low tidal volume (n=6); LV_T+ O_2: ventilated with LV_T with 50% oxygen (n=5).

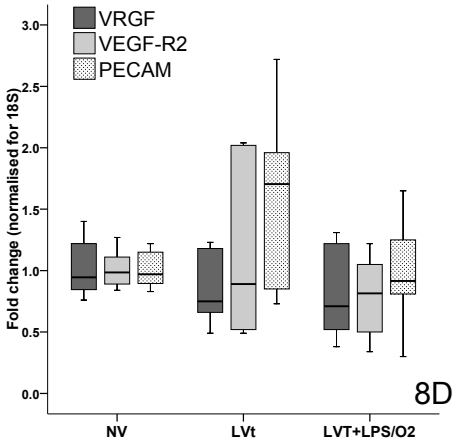

Figure 8D. Effect of exposure to low V_T ventilation (8 hours) with room air and low V_T ventilation (8 hours) with 50% oxygen after exposure to LPS on the expression of matrix genes controlling vascularisation in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles.

groups (Fig. 8A). VEGF expression was not changed by ventilation with ~3.5 ml.kg^{-1} and ~8.5 ml.kg^{-1}, but 8 h of ventilation with ~25 ml.kg was associated with significant increased levels of VEGF mRNA (Fig. 8A). Endothelial cell (PECAM) marker expression was only increased by ventilation with ~25 ml.kg^{-1}. *Series 2:* Interestingly, exposure to LPS and/or ventilation with 50% oxygen did not alter the expression of VEGF, VEGF-R2 and PECAM (Figs. 8B, C and D). *Series 3:* Prolonged (24 h) MV_T ventilation did increase

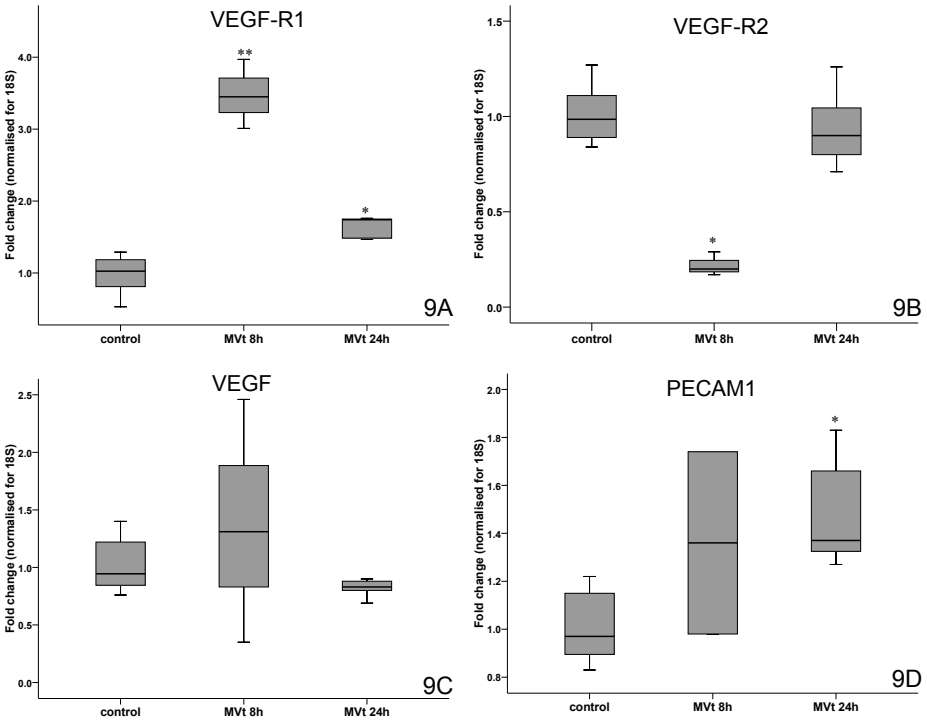

Figure 9. Effect of 8 and 24 hours of moderate V_T ventilation with room air and low V_T on the expression of matrix genes controlling vascularisation in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. **A)** *p < 0.05 *vs.* NV; **p < 0.05 *vs*. NV, 24hours; **B)** *p < 0.05 *vs.* NV, 24hours; **D)***p < 0.05 *vs.* NV.

the expression of VEGF-R1 *versus* non-ventilated animals (Fig. 9A). Again, this alteration was not as explicit as after 8 h MV_T-ventilation (Fig. 9A). The expression of VEGF and VEGF-R2 was not altered (Figs 9B, C). The expression of PECAM was significantly increased after 24 h MV_T-ventilation (Fig. 9D).

Vessel count: In animal models of mild BPD, the arrest in alveolar development is accompanied by a paucity of pulmonary capillaries. Small vessels are defined by vessels 20–65 μm in diameter. Ventilation with different tidal volumes for 8 h did not affect the vessel density (Table 3A). However, after 24 h MV_T ventilation of 7-day old rats a significant decrease in number of small vessels was observed *versus* non-ventilates 8-day old rats. The number of small vessels was not different from the number of small vessels in non-ventilated 7-day old rats (Table 3B).

TABLE 3A. Effect of low (LV_T), moderate (MV_T) and high (HV_T) ventilation for 8 hours on the vessel density in lungs of 7-day old rats.

	Tidal Volume			
Vessel density	NV (n=8)	LV_T 8h (n=4)	MV_T 8h(n=4)	HV_T 8h (n=4)
Vessels < 20 µm	23.7 ± 2.6	26.5 ± 3.5	22.7 ± 4.7	21.8 ± 2.9
Vessels 20-60 µm	25.0 ± 7.9	28.5 ± 2.6	21.0 ± 9.7	22.8 ± 4.6
Vessels > 60 µm	48.7 ± 7.0	55.0 ± 6.1	43.7 ± 8.8	44.6 ± 5.1

Vessel density per 30 images (X40 high power fields). Values represent mean ± SD.

TABLE 3B. Effect of prolonged (24h) ventilation on the vessel density in lungs of 7-day old rats.

	Tidal Volume		
Vessel density	NV day 7(n=4)	MV_T 24h (n=4)	NV day 8 (n=4)
Vessels < 20 µm	29.5 ± 3.1	39.3 ± 3.8*§	23.7± 2.6
Vessels 20-60 µm	11.3 ± 2.5§	12.3 ± 3.5§	25.0 ± 7.9
Vessels > 60 µm	40.8 ± 5.0	51.7 ± 5.0*	48.7 ± 7.0

Vessel density per 30 images (X40 high power fields). Values represent mean ± SD.
* $p < 0.05$ vs. control day 7, §$P < 0.05$ vs. control day 8.

DISCUSSION

Arrested lung growth is one of the hallmarks of BPD. In the present study, we focused on the effect of mechanical ventilation on pulmonary expression of genes that regulate formation of alveoli and blood vessels in lungs of newborn rats. Because in rats, lungs at birth have a saccular appearance and alveolarization occurs exclusively postnatal, this model is relevant to the premature born infants developing BPD, although we acknowledge the major differences between mechanically ventilated newborn rats and premature born infants (18). Herein, we demonstrated an alteration in expression of molecules involved in alveolarization and angiogenesis in relation to tidal volume and duration of ventilation.

Mechanical ventilation and expression of genes implicated in alveolarization

Formation of new alveoli during the first phase of alveolarization ((postnatal days 4-21 in rat), starts with the development of new septa from immature preexisting septa. Elastogenesis is an essential step to this process of alveolar septation (24). Elastin is synthesized as tropoelastin. Both fibulin5 and lysyl oxidase play a key role during the assembly of tropoelastin into elastin fibers. Fibulin5 and elastin null mice exhibit abnormal alveolar development (24, 25). Lungs of LOX null mice display impaired development of distal and proximal airways with dilatation of the alveolar walls, sacs and primitive alveolar structures (25). Mechanical forces have been shown to induce

mRNA expression of tropoelastin *in vitro* (26) and *in vivo* (27). It has been suggested that ventilation with LV_T is less injurious. We previously reported decreased alveolar development in newborn rats after 12 and 24 h of mechanical ventilation, but not after 8 h of mechanical ventilation (15, 18). In accordance with these data we found in the present study that ventilation with LV_T and MV_T for 8 h did hardly influence the expression of tropoelastin, lysyl oxidase and fibulin5. Injurious ventilation (HV_T) increased the gene expression of all three elastogenic genes, in line with previous reports showing up-regulation of these genes during lung injury repair (28-30). A recent study has also reported increased gene expression of tropoelastin and lysyl oxidase, but not fibulin5 after 8 h ventilation of 2-4 days old mice (31). However, since tidal volume settings were not reported it is difficult to compare those data to ours. Next we studied the effect of eight hours LV_T ventilation combined with a systemic inflammation and/or oxygen and a prolonged period of MV_T ventilation for 12 and 24 h. LV_T ventilation (8 h) with 50% oxygen and MV_T ventilation for (12 h) caused a significant increase in fibulin5 mRNA, while LV_T ventilation (8 h) with room air or oxygen after pre-exposure to LPS and MV_T ventilation for 24 h reduced the mRNA levels of fibulin5. MV_T ventilation for 12 hours increased mRNA expression of tropoelastin and lysyl oxidase. After 24 hours of MV_T ventilation the increase of expression of these genes was less explicit. In newborn mice ventilated for 24 h with 40% oxygen, only the elastin assembly proteins fibulin5 and emilin-1 were decreased and the protein content of tropoelastin in the lung was increased (31). Our data demonstrate that inflammation accelerates the ventilation-induced effect on fibulin5 gene expression. Short-term ventilation after pre-exposure to LPS had the same effect on fibulin5 gene expression of as long-term ventilation with room air without pre-exposure to LPS. The influence of inflammation on fibulin5 expression seems to have more impact than 50% oxygen. The up-regulation of fibulin5 after LV_T ventilation (8 h) with oxygen is completely abolished after pre-exposure to LPS. For normal elastic fiber formation molecular interactions of tropoelastin and several extracellular matrix proteins, including fibulin5 and lysyl oxidase, are essential. Lysyl oxidase plays a critical role in the formation and repair of the extracellular matrix (ECM) by oxidizing lysine residues in elastin and collagen. As a result, covalent crosslinkages which stabilize these fibrous proteins are formed (32). Fibulin5 is an ECM protein that can bind integrins. Its role seems to be anchorage of elastic fibres to cells, thereby stabilizing and organizing elastic fibres in the lung (33). It is reasonable to assume that normal elastic fiber formation requires a certain balance between both assembly molecules (lysyl oxidase and-fibulin5) and tropoelastin. During the first 12 h of MV_T ventilation we found increases in tropoelastin, lysyl oxidase, and fibulin5 gene expression. Interestingly, while expression of both tropoelastin and lysyl oxidase were still increased after 24 h of MV_T, expression of fibulin5 was decreased when compared to nonventilated controls. These data suggests that fibulin5 is prob-

ably one of the most important elastin assembly proteins, responsible for dysregulated elastin synthesis due to mechanical ventilation during the first 24 h.

ECM glycoprotein TN-C plays a morphoregulatory role during development and tissue remodeling. Fetal lungs of TN-C deficient mice have enlarged airspaces (34). In agreement with a previous study using newborn mice (35) we observed a significant down-regulation of TN-C expression after ventilation with low to moderate tidal volumes, with or without oxygen and/or pre-exposition to LPS. Both prolonged (24 h) MV_T ventilation with room air and LV_T-ventilation (8h) with oxygen after pre-exposure to LPS had the most negative impact on TN-C expression. Several findings support the idea that pulmonary TN-C is a mechanosensitive gene. Cyclic mechanical stretch (elongation 10% for 48 h) of human lung fibroblasts reduced TN-C expression and decreased differentiation into ECM producing myofibroblasts (36). In a murine model of pulmonary hypoplasia with co-existent diaphragmatic hernia TN-C expression is down-regulated (37). In contrast, cyclic mechanical strain of human airway smooth muscle cells (17–18.5% elongation for 1-5 days) has been shown to increase TN-C expression (38). Furthermore, TN-C is highly expressed in autopsied babies with BPD (39). Recent analysis of gene expression in repairing lungs of naphthalene-exposed mice showed a transient increase of TN-C expression in the lung whereas abortive repair in a transgenic model allowing ablation of all reparative cells resulted in a progressive accumulation of TN-C (49). Thus, the increase in TN-C expression during HV_T ventilation fits with a lung injury repair response. In contrast, the down-regulation of TN-C after 8-24 h of mechanical ventilation with low to moderate tidal volumes may negatively influence alveolar development, although we did not find significant differences at the TN-C protein level.

Matrix metalloproteinases (MMPs) represent a family of zinc-dependent proteases that degrade the ECM. These proteases are secreted by endothelial cells, alveolar cells, macrophages, fibroblasts, and other connective tissue cells (40). They have been shown to play a role in airway remodeling (41). By breaking down ECM proteins they facilitate cell migration, which is essential for normal wound repair (42). Since mechanical ventilation triggers the expression of acute phase cytokines (15) it is not surprising that MMPs are reportedly elevated in BPD (19-22). We did find an increase in Mmp9 message after 12 h of mechanical ventilation, however, the exact ECM targets are yet unknown.

Mechanical ventilation and expression of genes implicated in angiogenesis

Ample evidence suggests that abnormal vascularization may contribute to disrupted alveolar development. Especially reduced signaling through angiogenic VEGF and PDGF pathways appear to affect alveologenesis. Blocking PDGF receptor signaling in neonatal rats causes abnormal alveolar development (43). Expression of VEGF and

its receptors are decreased in premature lambs, baboons and human infants, who developed BPD after a prolonged period of mechanical ventilation with oxygen (9, 44, 45). In the present study, non-injurious (LV_T and MV_T) ventilation with room air had no affect on VEGF expression. Even prolonged ventilation (24 h) and ventilation (8 h) with oxygen and pre-exposure to LPS did not change the expression of VEGF. We only observed a significant decrease of VEGF-R2 expression after MV_T ventilation. Although VEGF-R1 expression was up-regulated with LV_T and MV_T ventilation for 8 h, it is believed that VEGF-R2 mediates the major growth and permeability actions of VEGF. Thus, LV_T ventilation appears to have has less influence on lung vascular development in comparison with MV_T ventilation. Our finding of reduced PDGF-B expression after MV_T and HV_T ventilation, but not LV_T ventilation, supports this conclusion. PDGF-B/PDGF-Rβ signaling has been shown to promote proliferation of vascular smooth muscle cells and pericytes during their recruitment to new vessels (46). Only after 24 h of MV_T ventilation we observed a decrease in small vessel density compared to non-ventilated controls. Proper PDGF-A/PDGF-Rα signaling is crucial for alveolar septation (47). In contrast with a previous study using newborn mice (35) prolonged ventilation, independent of V_T, did not reduce the expression of PDGF-A in neonatal rats. Interestingly, independent of tidal volume, PDGF-Rα expression was increased by ventilation. An increase in PDGF-Rα has been shown to be up-regulated prior to the development of fibroproliferative lung lesions (48), while a decrease in PDGF-Rα expressing lung fibroblasts coincided with alveolar thining (49).

In conclusion, MV_T ventilation alters the expression of genes involved in alveolarization and angiogenesis in newborn rats. Our data suggest an imbalance between the expression of fibulin5 and tropoelastin as the first trigger for dysregulated elastin formation during the first 24 h of mechanical ventilation. This ventilatory negative effect on Fibulin5 and tropoelastin expression is accelerated by a systemic inflammation. We also show that altered PDGF and VEGF receptor expression after MV_T-ventilation affects pulmonary vessel formation. LV_T ventilation was protective, suggesting that the negative effect of ventilation on angiogenesis is tidal volume dependent. We are the first to demonstrate these alterations in gene expression in relation to tidal volume and duration of ventilation. This may be important for future therapeutic strategies to prevent BPD.

REFERENCES

1. Vrijlandt EJ, Gerritsen J, Boezen HM, Grevink RG, Duiverman EJ. Lung function and exercise capacity in young adults born prematurely. Am J Respir Crit Care Med. 2006 Apr 15;173(8):890-6.
2. Doyle LW, Faber B, Callanan C, Freezer N, Ford GW, Davis NM. Bronchopulmonary dysplasia in very low birth weight subjects and lung function in late adolescence. Pediatrics. 2006 Jul;118(1):108-13.
3. Northway WH, Jr., Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med. 1967 Feb 16; 276(7):357-68.
4. Greenlee KJ, Werb Z, Kheradmand F. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. Physiol Rev. 2007 Jan;87(1):69-98.
5. van Tuyl M, Post M. From fruitflies to mammals: mechanisms of signalling via the Sonic hedgehog pathway in lung development. Respir Res. 2000;1(1):30-5.
6. Margraf LR, Tomashefski JF, Jr., Bruce MC, Dahms BB. Morphometric analysis of the lung in bronchopulmonary dysplasia. Am Rev Respir Dis. 1991 Feb;143(2):391-400.
7. Hislop AA, Wigglesworth JS, Desai R, Aber V. The effects of preterm delivery and mechanical ventilation on human lung growth. Early Hum Dev. 1987 May;15(3):147-64.
8. Pierce RA, Albertine KH, Starcher BC, Bohnsack JF, Carlton DP, Bland RD. Chronic lung injury in preterm lambs: disordered pulmonary elastin deposition. Am J Physiol. 1997 Mar; 272(3 Pt 1):L452-60.
9. Bland RD, Xu L, Ertsey R, Rabinovitch M, Albertine KH, Wynn KA, et al. Dysregulation of pulmonary elastin synthesis and assembly in preterm lambs with chronic lung disease. Am J Physiol Lung Cell Mol Physiol. 2007 Jun;292(6):L1370-84.
10. Hilgendorff A, Parai K, Ertsey R, Jain N, Navarro EF, Peterson JL, et al. Inhibiting Lung Elastase Activity Enables Lung Growth in Mechanically Ventilated Newborn Mice. Am J Respir Crit Care Med. 2011 May 11.
11. Yuan S, Hannam V, Belcastro R, Cartel N, Cabacungan J, Wang J, et al. A role for platelet-derived growth factor-BB in rat postpneumonectomy compensatory lung growth. Pediatr Res. 2002 Jul;52(1):25-33.
12. Jankov RP, Kantores C, Belcastro R, Yi S, Ridsdale RA, Post M, et al. A role for platelet-derived growth factor beta-receptor in a newborn rat model of endothelin-mediated pulmonary vascular remodeling. Am J Physiol Lung Cell Mol Physiol. 2005 Jun;288(6):L1162-70.
13. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 1989 Dec 8;246(4935):1306-9.
14. Roth-Kleiner M, Ridsdale R, Cao L, Kuliszewski M, Tseu I, McKerlie C, et al. Lipopolysaccharide exposure modifies high tidal volume ventilation-induced proinflammatory mediator expression in newborn rat lungs. Pediatr Res. 2007 Feb;61(2):191-6.
15. Kroon AA, Wang J, Huang Z, Cao L, Kuliszewski M, Post M. Inflammatory response to oxygen and endotoxin in newborn rat lung ventilated with low tidal volume. Pediatr Res. 2010 Jul;68(1):63-9.
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.
17. Masood A, Yi M, Lau M, Belcastro R, Shek S, Pan J, et al. Therapeutic effects of hypercapnia on chronic lung injury and vascular remodeling in neonatal rats. Am J Physiol Lung Cell Mol Physiol. 2009 Nov;297(5):L920-30.

18. Kroon AA, Wang J, Kavanagh B, Huang Z, Kuliszewski M, van Goudoever JB, et al. Prolonged mechanical ventilation induces cell cycle arrest in newborn rat lung. PLoS One. 2011;6(2):e16910.
19. Ekekezie, II, Thibeault DW, Simon SD, Norberg M, Merrill JD, Ballard RA, et al. Low levels of tissue inhibitors of metalloproteinases with a high matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio are present in tracheal aspirate fluids of infants who develop chronic lung disease. Pediatrics. 2004 Jun;113(6):1709-14.
20. Schock BC, Sweet DG, Ennis M, Warner JA, Young IS, Halliday HL. Oxidative stress and increased type-IV collagenase levels in bronchoalveolar lavage fluid from newborn babies. Pediatr Res. 2001 Jul;50(1):29-33.
21. Tambunting F, Beharry KD, Hartleroad J, Waltzman J, Stavitsky Y, Modanlou HD. Increased lung matrix metalloproteinase-9 levels in extremely premature baboons with bronchopulmonary dysplasia. Pediatr Pulmonol. 2005 Jan;39(1):5-14.
22. Radomski A, Sawicki G, Olson DM, Radomski MW. The role of nitric oxide and metalloproteinases in the pathogenesis of hyperoxia-induced lung injury in newborn rats. Br J Pharmacol. 1998 Dec;125(7):1455-62.
23. Thebaud B, Ladha F, Michelakis ED, Sawicka M, Thurston G, Eaton F, et al. Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. Circulation. 2005 Oct 18;112(16):2477-86.
24. Wendel DP, Taylor DG, Albertine KH, Keating MT, Li DY. Impaired distal airway development in mice lacking elastin. Am J Respir Cell Mol Biol. 2000 Sep;23(3):320-6.
25. Maki JM, Sormunen R, Lippo S, Kaarteenaho-Wiik R, Soininen R, Myllyharju J. Lysyl oxidase is essential for normal development and function of the respiratory system and for the integrity of elastic and collagen fibers in various tissues. Am J Pathol. 2005 Oct;167(4):927-36.
26. Nakamura T, Liu M, Mourgeon E, Slutsky A, Post M. Mechanical strain and dexamethasone selectively increase surfactant protein C and tropoelastin gene expression. Am J Physiol Lung Cell Mol Physiol. 2000 May;278(5):L974-80.
27. Joyce BJ, Wallace MJ, Pierce RA, Harding R, Hooper SB. Sustained changes in lung expansion alter tropoelastin mRNA levels and elastin content in fetal sheep lungs. Am J Physiol Lung Cell Mol Physiol. 2003 Apr;284(4):L643-9.
28. Kuang PP, Goldstein RH, Liu Y, Rishikof DC, Jean JC, Joyce-Brady M. Coordinate expression of fibulin-5/DANCE and elastin during lung injury repair. Am J Physiol Lung Cell Mol Physiol. 2003 Nov;285(5):L1147-52.
29. Rocco PR, Negri EM, Kurtz PM, Vasconcellos FP, Silva GH, Capelozzi VL, et al. Lung tissue mechanics and extracellular matrix remodeling in acute lung injury. Am J Respir Crit Care Med. 2001 Sep 15;164(6):1067-71.
30. Kumarasamy A, Schmitt I, Nave AH, Reiss I, van der Horst I, Dony E, et al. Lysyl oxidase activity is dysregulated during impaired alveolarization of mouse and human lungs. Am J Respir Crit Care Med. 2009 Dec 15;180(12):1239-52.
31. Bland RD, Ertsey R, Mokres LM, Xu L, Jacobson BE, Jiang S, et al. Mechanical ventilation uncouples synthesis and assembly of elastin and increases apoptosis in lungs of newborn mice. Prelude to defective alveolar septation during lung development? Am J Physiol Lung Cell Mol Physiol. 2008 Jan;294(1):L3-14.

32. Lucero HA, Kagan HM. Lysyl oxidase: an oxidative enzyme and effector of cell function. Cell Mol Life Sci. 2006 Oct;63(19-20):2304-16.
33. Nakamura T, Lozano PR, Ikeda Y, Iwanaga Y, Hinek A, Minamisawa S, et al. Fibulin-5/DANCE is essential for elastogenesis in vivo. Nature. 2002 Jan 10;415(6868):171-5.
34. Roth-Kleiner M, Hirsch E, Schittny JC. Fetal lungs of tenascin-C-deficient mice grow well, but branch poorly in organ culture. Am J Respir Cell Mol Biol. 2004 Mar;30(3):360-6.
35. Bland RD, Mokres LM, Ertsey R, Jacobson BE, Jiang S, Rabinovitch M, et al. Mechanical ventilation with 40% oxygen reduces pulmonary expression of genes that regulate lung development and impairs alveolar septation in newborn mice. Am J Physiol Lung Cell Mol Physiol. 2007 Nov;293(5):L1099-110.
36. Blaauboer ME, Smit TH, Hanemaaijer R, Stoop R, Everts V. Cyclic mechanical stretch reduces myofibroblast differentiation of primary lung fibroblasts. Biochem Biophys Res Commun. 2011 Jan 7;404(1):23-7.
37. Chinoy MR, Miller SA. Relevance of tenascin-C and matrix metalloproteinases in vascular abnormalities in murine hypoplastic lungs. Biol Neonate. 2006;90(3):185-96.
38. Hasaneen NA, Zucker S, Cao J, Chiarelli C, Panettieri RA, Foda HD. Cyclic mechanical strain-induced proliferation and migration of human airway smooth muscle cells: role of EMMPRIN and MMPs. FASEB J. 2005 Sep;19(11):1507-9.
39. Kaarteenaho-Wiik R, Kinnula VL, Herva R, Soini Y, Pollanen R, Paakko P. Tenascin-C is highly expressed in respiratory distress syndrome and bronchopulmonary dysplasia. J Histochem Cytochem. 2002 Mar;50(3):423-31.
40. Nagase H, Woessner JF, Jr. Matrix metalloproteinases. J Biol Chem. 1999 Jul 30;274(31):21491-4.
41. Vu TH. Don't mess with the matrix. Nat Genet. 2001 Jul;28(3):202-3.
42. Chen P, Parks WC. Role of matrix metalloproteinases in epithelial migration. J Cell Biochem. 2009 Dec 15;108(6):1233-43.
43. Lau M, Masood A, Yi M, Belcastro R, Li J, Tanswell AK. Long-term failure of alveologenesis after an early short-term exposure to a PDGF-receptor antagonist. Am J Physiol Lung Cell Mol Physiol. 2011 Apr;300(4):L534-47.
44. Bhatt AJ, Pryhuber GS, Huyck H, Watkins RH, Metlay LA, Maniscalco WM. Disrupted pulmonary vasculature and decreased vascular endothelial growth factor, Flt-1, and TIE-2 in human infants dying with bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 1):1971-80.
45. Maniscalco WM, Watkins RH, Pryhuber GS, Bhatt A, Shea C, Huyck H. Angiogenic factors and alveolar vasculature: development and alterations by injury in very premature baboons. Am J Physiol Lung Cell Mol Physiol. 2002 Apr;282(4):L811-23.
46. Betsholtz C. Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev. 2004 Aug;15(4):215-28.
47. Hoch RV, Soriano P. Roles of PDGF in animal development. Development. 2003 Oct;130(20):4769-84.
48. Lasky JA, Tonthat B, Liu JY, Friedman M, Brody AR. Upregulation of the PDGF-alpha receptor precedes asbestos-induced lung fibrosis in rats. Am J Respir Crit Care Med. 1998 May;157(5 Pt 1):1652-7.
49. McGowan SE, McCoy DM. Fibroblasts expressing PDGF-receptor-alpha diminish during alveolar septal thinning in mice. Pediatr Res. 2011 Jul;70(1):44-9.

Chapter 8

General discussion

INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a chronic lung disease, one of the most important sequelae of oxygen and ventilation-mediated injuries following a premature birth. The pulmonary impairment begins *in utero,* progresses postnatally and continues into later childhood and adulthood. Treatment with antenatal corticosteroids, introduction of protective ventilation strategies, surfactant replacement therapy, progress in nutritional support and better nursing care has improved survival of premature born infants with Respiratory Distress Syndrome, but the incidence of BPD has hardly changed. BPD is characterized by an arrest in alveolar development: alveoli are fewer and larger, and the vasculature is dysmorphic. BPD remains a major complication in premature infants, who are mechanically ventilated with oxygen; most likely due to the ever-changing limit of viability resulting in higher survival rates of infants born at gestational age less then 26 weeks. Both mechanical ventilation and oxygen interfere with alveolar and vascular development. The main goal of this thesis was to identify molecular pathways that are activated by mechanical ventilation and interfere with normal lung development. To investigate these underlying pathogenic pathways we used a newborn rat model.

LIMITATIONS OF THE NEWBORN RAT MODEL

Morphologic and quantitative studies of the rat lung have greatly contributed to the understanding of the formation of alveoli. However, major differences exist between neonatal rats and premature infants. First, neonatal rats that receive mechanical ventilation are at term and consequently have a more developed surfactant system. With the use of stable isotopes it has been shown that the *de novo* synthesis and turnover rates of surfactant in preterm infants with respiratory distress syndrome are very low (1). Secondly, the anti-oxidant system in neonatal rats is more mature. Human studies have demonstrated that preterm infants produce high amounts of reactive oxygen species (ROS), while lacking appropriate amounts of antioxidant enzymes, including superoxide dismutase and glutathione peroxidase (2). The antioxidant system is upregulated during the last 15% of gestation (3). A major advantage is that at birth rat lungs mimic the saccular lung of very premature patients and alveolar formation occurs exclusively postnatally within a very short period of about 2 weeks (4). There are other animal models to study alveolarization, but some are very expensive (premature baboons and sheep) while others are technically a challenge to ventilate (newborn mice). Hence, despite its shortcomings, the newborn rat is an appropriate model to study ventilatory-induced pathways leading to arrested alveolarization. This is relevant to premature born infants at risk to develop BPD.

EFFECT OF DIFFERENT TIDAL VOLUMES AND DURATION OF MECHANICAL VENTILATION ON INFLAMMATORY RESPONSE

In **chapter 1** the different mechanisms of ventilator-induced lung injury (VILI) are described. High peak inspiratory pressures and repetitive end-expiratory collapse are considered to be important determinants of VILI. That's why high frequency oscillatory ventilation (HFOV) has been introduced in the neonatal care. HFOV delivers small tidal volumes at rapid rates. Multiple studies in animals have suggested that this mode of ventilation is less injurious compared to conventional ventilation. HFOV improved early lung function and decreased inflammation (5). However, clinical trials comparing HFOV with conventional ventilation showed only a very small reduction in BPD with HFOV and no longterm benefits (6, 7). In baboons with BPD impaired alveolarization and capillary development occurred in spite of appropriate oxygenation (FiO2: 0.2-0.5) and use of ventilator strategies to prevent volutrauma (8). Thus even minute stretching of a developing lung is enough to cause injury. In **chapter 3** we describe that both low V_T and moderate V_T ventilation with room air for 8 h induced an inflammatory response. The expression of IL-6 and CXCL-2 were increased. The expression of these genes was further increased following low V_T ventilation with 50% oxygen. Il-6 and CXCL-2 activate and attract leukocytes that are implicated in the pathogenesis of BPD (9, 10). In **chapter 7** we describe the effect of different tidal volumes on the expression of genes involved in alveolarization. We found an almost similar change in expression of these genes after 8 h of low or moderate V_T ventilation. Expression of tropoelastin and lysyl oxidase was increased while that of tenascin-C was decreased compared to nonventilated controls. Lysyl oxidase and tropoelastin message levels peaked at 12 hours of ventilation and remained elevated at 24 hours. In contrast, fibulin5 expression was increased after 12 hours of ventilation but decreased compared to nonventilated controls at the time (24 hours) we observed a disturbed elastin deposition. Short-term moderate V_T ventilation after pre-exposure to LPS had the same negative effect on fibulin5 expression as long-term ventilation with room air without pre-exposure to LPS. Although our prolonged (24 hrs) ventilation used a moderate V_T, it is reasonable to assume that the changes in gene expression after prolonged ventilation with low V_T will be the same as those seen with moderate V_T, considering the changes in gene expression after 8 h. Uncoupled elastin synthesis and assembly could explain decreased septation (alveolarization), as seen in BPD lungs (11).

These data may explain why randomised controlled trials did not show beneficial effect of protective ventilation (HFOV) in preventing BPD. However, we have to consider the following issues: (a) the limitations of the model as described above; (b) during the period with low and moderate tidal volume ventilation it was not possible to optimize the lung volume ventilation strategy to recruit alveoli, eliminate atelectasis or prevent

overdistension because of lack of continuous measurement of O_2 saturation or $PaO_{2;}$ ventilation strategies which have demonstrated to reduce VILI (12); (c) animals were not allowed to recover after the ventilation period.

It still has to be elucidated if the disturbed elastogenesis is a direct result of ventilation-triggered cell stretch or a secondary effect of increased expression of pro-inflammatory cytokines and chemokines. A systemic inflammation in fetal sheep increased the concentration of IL-6 in bronchoalveolar lavage, together with disturbed deposition of elastin in the lungs (13). This finding and our data suggest that the IL-6 pathway may be relevant in disturbed elastin deposition.

MECHANICAL VENTILATION INDUCES APOPTOSIS AND CELL CYCLE ARREST

Normal lung development is a highly orchestrated process of growth, differentiation and programmed cell death (14, 15). During normal lung development three peaks of apoptosis, preceded by peaks of cell proliferation are described (**chapter 1**). *In vitro* studies using isolated lung cells have demonstrated that mechanical overstretch inhibits proliferation and induces apoptosis (15, 16). Only stretch-induced apoptosis, but not stretch-induced cell cycle arrest, has been demonstrated *in vivo* (11). Such interference in cell proliferation and programmed cell death may affect alveolar formation in the developing lung. Consequently it is reasonable to hypothesize that dysfunctional apoptotic and cell proliferation mechanisms contribute to the pathophysiology of BPD. Our studies provide evidence for this hypothesis. After prolonged (24 hours) mechanical ventilation we found increased apoptosis and inhibited proliferation of lung cells together with an arrest in alveolar development.

Different pathways regulate both apoptosis and proliferation of cells. For a better understanding of the pathogenesis of BPD, it is essential to know which signaling pathway are involved. In **chapter 5** we show that prolonged mechanical ventilation for 24 h increased the number of TUNEL and cleaved caspase-3 positive lung cells *via* activation of the FasL/Fas (extrinsic apoptotic) pathway. This was confirmed by stretching of primary isolated rat fetal lung epithelial cells. Activation of the extrinsic apoptotic pathway was demonstrated by increased activity of caspase-8, while caspase-9 activity (intrinsic apoptotic pathway) was not increased. Also the expression of FasL mRNA and protein was increased. Inhibition by universal inhibitors of caspases abrogated stretch-induced caspase-3 activation and a neutralizing anti-rat FasL monoclonal antibody attenuated the stretch-induced levels of cleaved caspases-3 and -8. It is plausible that the extrinsic apoptotic pathway via the FasL/Fas pathway is directly activated by stretch. Tracheal occlusion of fetal rabbits resulted in an increase of FasL in alveolar type II cells, together with increased apoptosis of these cells (17). Other triggers for activation of the

extrinsic apoptotic pathway by mechanical ventilation are pro-inflammatory cytokines/chemokines. Again IL-6 seems to play an important part. IL-6 has been shown to induce apoptosis in some models, but it also exerts anti-apoptotic effect in others (18, 19). IL-6 is linked with the pathogenesis of emphysema. In IL-6 deficient mice treated with pancreatic elastase, hyperoxia and sigarette smoke, the increase of apoptotic cells was suppressed (18, 20, 21). Unfortunately, the FasL/Fas pathway was not studied. Although IL-6's involvement in ventilation/stretch-induced apoptosis remains to be determined, our finding of increased expression of IL-6 after 8 h of mechanical ventilation (**chapter 3**) is compatible with such a role. However, besides IL-6, mechanical ventilation upregulates other pro-inflammatory mediators (IL-1β, IL-8 and TGF-β), that exhibit pro-apoptotic properties.

In **chapter 6** the effect of prolonged mechanical ventilation on lung cell proliferation is elucidated. The response of pulmonary epithelial cells to mechanical stretch *in vitro* has been shown to vary considerably depending on the cell type, culture conditions, pattern, and duration of stretch exposure (22). Mechanical stretch simulating normal fetal breathing movements increased type II cell proliferation, indicating that mechanical strain is necessary for normal lung growth (23, 24). From *in vitro* studies it is known that mechanical stretch and oxygen can also inhibit lung cell proliferation (25, 26). We are the first to describe that ventilation for 24 h inhibits uptake of BrdU in lung cells and decreases expression of Cyclin D1 en E1 mRNA and protein. Before a cell can proliferate, it needs to pass through four non-overlapping phases (**chapter 1**). We give evidence that the ventilation-induced arrest in lung cell proliferation takes place in G_1 phase. In this phase the cell is preparing for DNA synthesis. Expression and nuclear localization of Cdk inhibitor proteins $p27^{Kip1}$ and $p57^{Kip2}$ was increased. This prevents binding of Cyclin D1 and E1 to their CDK partner, which is essential for transition from G_1 to S phase of the cell cycle. It is not known how mechanical strain influences this binding. We could not identify transforming growth factor-β (TGF-β) as a trigger in our study. We do know that TGF-β can prevent the decline of $p27^{Kip1}$mRNA expression. TGFβ-treated cells have $p27^{Kip1}$ mRNA levels equal to those of proliferating cells (27). In the bronchoalveolar lavage fluid of preterm infants increased levels of TGF-β have been found in those patients who developed BPD (28, 29). In contrast, high tidal volume ventilation for 3 hours did not increase TGF-β content in newborn rat lung (30) and stretching subcutaneous tissue did decrease TGF-β protein (31). Thus, it is plausible that the level of TGF-β expression is important for long-term outcome by determining the intensity of tissue remodeling and repair (32). We found an arrest in lung cell proliferation, with no change in TGF-β expression, suggesting that after 24 h of moderate tidal volume ventilation remodeling or repair of lung tissue has not yet started. Undoubtedly this thesis and future research will bring us a step closer to elucidate the pathogenesis of BPD.

FUTURE PERSPECTIVES

The general aim of this thesis was to identify molecular pathways that are activated by mechanical ventilation and interfere with normal lung development. We were able to unmask two pathways in ventilated newborn rats. First, mechanical ventilation induces apoptosis of lung cell via the FasL/Fas pathway. Secondly, mechanical ventilation induces an arrest in lung cell proliferation by increased expression and nuclear localization of Cdk inhibitors from the Kip family. Normal lung development is a highly orchestrated process of growth, differentiation and programmed cell death. The imbalance we found between lung cell proliferation and programmed cell death may be the key to the disturbed alveolar formation in the developing lung.

A logical next step will be to study if inhibition of the FasL/Fas pathway and targeting protein $p27^{Kip1}$ will prevent or at least attenuate the arrest in alveolarization. The ultimate goal will be to develop treatment strategies to prevent this ventilator-induced injury for the premature born infants based on our findings. However, treatment strategies based on inhibiting apoptosis or stimulating proliferation do carry the potential harm of inhibiting physiological apoptosis and inducing/enhancing proliferation in other cells such as fibroblasts and neutrophils. This can be even more harmful for the developing lung. Hence, a better understanding of the role of apoptosis and proliferation in the pathogenesis of ventilator-induced alterations in lung development and the signaling cascades are necessary before therapeutic strategies can be developed for the treatment.

Another promising direction for research will be stem/progenitor cell therapy. Studies in the adult lung suggest that bone marrow-derived stem cells (BMCs) can prevent adult lung injury. *In vitro* BMSCs prevent alveolar growth arrest in a BPD model. They adopt immunophenotypic and ultrastructural characteristics of alveolar type II cells (33). Also an increased number of circulating endothelial progenitor cells (EPCs) in patients with acute lung injury is correlated with improved survival (34). Given the fact that disrupted alveolarization is associated with a dysmorphic microvasculature and the importance of lung vasculature for repair after lung injury, EPCs may have therapeutic potential. Umbilical cord blood represents an important source for stem cells. The establishment of a biobank in which umbilical cord and cord blood can be collected will be a first step in a better understanding of stem cell biology and its therapeutical potential. At the same time trachea aspirates can be collected to find early biomarkers and potential mediators of ventilation-induced lung injury in preterm infants.

Besides basic research, clinical studies are needed. Good follow-up studies of infants with BPD are lacking. Adverse health consequences of BPD include long-term respiratory morbidity such as asthma and emphysema, pulmonary hypertension, undernutrition, growth failure, cognitive impairment, cerebral palsy and neurodevelopmental

deficits. Until now there is no specific follow-up program and no guidelines for therapy. This requires a multidisciplinairy approach. Finally, to reduce the adverse effects associated with intubation and mechanical ventilation, non-invasive ventilation looks promising. There is evidence that non-invasive ventilation improves gas exchange and reduces extubation failure after mechanical ventilation in preterm born infants, but multicenter clinical trials are needed to demonstrate of this mode of respiratory support will decrease the incidence of BPD.

REFERENCES

1. Bunt JE, Zimmermann LJ, Wattimena JL, van Beek RH, Sauer PJ, Carnielli VP. Endogenous surfactant turnover in preterm infants measured with stable isotopes. Am J Respir Crit Care Med. 1998 Mar;157(3 Pt 1):810-4.
2. Nassi N, Ponziani V, Becatti M, Galvan P, Donzelli G. Anti-oxidant enzymes and related elements in term and preterm newborns. Pediatr Int. 2009 Apr;51(2):183-7.
3. Davis JM, Auten RL. Maturation of the antioxidant system and the effects on preterm birth. Semin Fetal Neonatal Med. 2010 Aug;15(4):191-5.
4. Burri PH. Structural aspects of postnatal lung development - alveolar formation and growth. Biol Neonate. 2006;89(4):313-22.
5. Yoder BA, Siler-Khodr T, Winter VT, Coalson JJ. High-frequency oscillatory ventilation: effects on lung function, mechanics, and airway cytokines in the immature baboon model for neonatal chronic lung disease. Am J Respir Crit Care Med. 2000 Nov;162(5):1867-76.
6. Henderson-Smart DJ, Cools F, Bhuta T, Offringa M. Elective high frequency oscillatory ventilation versus conventional ventilation for acute pulmonary dysfunction in preterm infants. Cochrane Database Syst Rev. 2007(3):CD000104.
7. Courtney SE, Durand DJ, Asselin JM, Hudak ML, Aschner JL, Shoemaker CT. High-frequency oscillatory ventilation versus conventional mechanical ventilation for very-low-birth-weight infants. N Engl J Med. 2002 Aug 29;347(9):643-52.
8. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. Am J Respir Crit Care Med. 1999 Oct;160(4):1333-46.
9. Jobe AH, Ikegami M. Mechanisms initiating lung injury in the preterm. Early Hum Dev. 1998 Nov;53(1):81-94.
10. Yi M, Jankov RP, Belcastro R, Humes D, Copland I, Shek S, et al. Opposing effects of 60% oxygen and neutrophil influx on alveologenesis in the neonatal rat. Am J Respir Crit Care Med. 2004 Dec 1;170(11):1188-96.
11. Mokres LM, Parai K, Hilgendorff A, Ertsey R, Alvira CM, Rabinovitch M, et al. 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. Jan; 298(1):L23-35.
12. van Kaam AH, Haitsma JJ, De Jaegere A, van Aalderen WM, Kok JH, Lachmann B. Open lung ventilation improves gas exchange and attenuates secondary lung injury in a piglet model of meconium aspiration. Crit Care Med. 2004 Feb;32(2):443-9.
13. Kramer BW, Ladenburger A, Kunzmann S, Speer CP, Been JV, van Iwaarden JF, et al. Intravenous lipopolysaccharide-induced pulmonary maturation and structural changes in fetal sheep. Am J Obstet Gynecol. 2009 Feb;200(2):195 e1-10.
14. Luyet C, Burri PH, Schittny JC. Suppression of cell proliferation and programmed cell death by dexamethasone during postnatal lung development. Am J Physiol Lung Cell Mol Physiol. 2002 Mar;282(3):L477-83.
15. Scavo LM, Ertsey R, Chapin CJ, Allen L, Kitterman JA. Apoptosis in the development of rat and human fetal lungs. Am J Respir Cell Mol Biol. 1998 Jan;18(1):21-31.
16. Edwards YS, Sutherland LM, Power JH, Nicholas TE, Murray AW. Cyclic stretch induces both apoptosis and secretion in rat alveolar type II cells. FEBS Lett. 1999 Apr 1;448(1): 127-30.

17. De Paepe ME, Mao Q, Luks FI. Expression of apoptosis-related genes after fetal tracheal occlusion in rabbits. J Pediatr Surg. 2004 Nov;39(11):1616-25.
18. Ward NS, Waxman AB, Homer RJ, Mantell LL, Einarsson O, Du Y, et al. Interleukin-6-induced protection in hyperoxic acute lung injury. Am J Respir Cell Mol Biol. 2000 May; 22(5):535-42.
19. Oritani K, Tomiyama Y, Kincade PW, Aoyama K, Yokota T, Matsumura I, et al. Both Stat3-activation and Stat3-independent BCL2 downregulation are important for interleukin-6-induced apoptosis of 1A9-M cells. Blood. 1999 Feb 15;93(4):1346-54.
20. Tasaka S, Inoue K, Miyamoto K, Nakano Y, Kamata H, Shinoda H, et al. Role of interleukin-6 in elastase-induced lung inflammatory changes in mice. Exp Lung Res. 2010 Aug; 36(6):362-72.
21. Ruwanpura SM, McLeod L, Miller A, Jones J, Bozinovski S, Vlahos R, et al. Interleukin-6 promotes Pulmonary Emphysema Associated with Apoptosis in Mice. Am J Respir Cell Mol Biol. 2011 Feb 17.
22. Liu M, Xu J, Souza P, Tanswell B, Tanswell AK, Post M. The effect of mechanical strain on fetal rat lung cell proliferation: comparison of two- and three-dimensional culture systems. In Vitro Cell Dev Biol Anim. 1995 Dec;31(11):858-66.
23. Xu J, Liu M, Tanswell AK, Post M. Mesenchymal determination of mechanical strain-induced fetal lung cell proliferation. Am J Physiol. 1998 Sep;275(3 Pt 1):L545-50.
24. Nardo L, Maritz G, Harding R, Hooper SB. Changes in lung structure and cellular division induced by tracheal obstruction in fetal sheep. Exp Lung Res. 2000 Mar;26(2):105-19.
25. Sanchez-Esteban J, Wang Y, Cicchiello LA, Rubin LP. Cyclic mechanical stretch inhibits cell proliferation and induces apoptosis in fetal rat lung fibroblasts. Am J Physiol Lung Cell Mol Physiol. 2002 Mar;282(3):L448-56.
26. McAdams RM, Mustafa SB, Shenberger JS, Dixon PS, Henson BM, DiGeronimo RJ. Cyclic stretch attenuates effects of hyperoxia on cell proliferation and viability in human alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2006 Aug;291(2):L166-74.
27. Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, et al. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell. 1994 Jul 15;78(1):59-66.
28. Speer CP. Inflammation and bronchopulmonary dysplasia: a continuing story. Semin Fetal Neonatal Med. 2006 Oct;11(5):354-62.
29. Kotecha S, Wangoo A, Silverman M, Shaw RJ. Increase in the concentration of transforming growth factor beta-1 in bronchoalveolar lavage fluid before development of chronic lung disease of prematurity. J Pediatr. 1996 Apr;128(4):464-9.
30. Wu S, Capasso L, Lessa A, Peng J, Kasisomayajula K, Rodriguez M, et al. High tidal volume ventilation activates Smad2 and upregulates expression of connective tissue growth factor in newborn rat lung. Pediatr Res. 2008 Mar;63(3):245-50.
31. Bouffard NA, Cutroneo KR, Badger GJ, White SL, Buttolph TR, Ehrlich HP, et al. Tissue stretch decreases soluble TGF-beta1 and type-1 procollagen in mouse subcutaneous connective tissue: evidence from ex vivo and in vivo models. J Cell Physiol. 2008 Feb;214(2):389-95.
32. Bartram U, Speer CP. The role of transforming growth factor beta in lung development and disease. Chest. 2004 Feb;125(2):754-65.
33. van Haaften T, Byrne R, Bonnet S, Rochefort GY, Akabutu J, Bouchentouf M, et al. Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. Am J Respir Crit Care Med. 2009 Dec 1;180(11):1131-42.

34. Burnham EL, Taylor WR, Quyyumi AA, Rojas M, Brigham KL, Moss M. Increased circulating endothelial progenitor cells are associated with survival in acute lung injury. Am J Respir Crit Care Med. 2005 Oct 1;172(7):854-60.

Chapter 9

Summary

In **Chapter 1** – the general introduction – the current knowledge regarding BPD related to lung development and lung injury is discussed. The critical concept underlying the pathogenesis of BPD is that the lung is structurally immature in preterm born infants. Any antenatal or postnatal event that will take place during this period can adversely alter the developmental program of the immature lung. The pathological findings seen in BPD is the result of a complex interplay between perinatal insults (infection, chorioamnionitis), impairments in the premature lung (surfactant deficiency, less developed antioxidant system) and damage resulting from lifesaving therapies, such as mechanical ventilation and supplemental oxygen administration. Together they culminate in a chronic inflammation in the developing lung that impairs alveolarization and vascularization. Both processes are essential for normal lung development and without doubt play a role in the pathogenesis of BPD.

The aims of this thesis are described in **Chapter 2**.

In **Chapter 3** we assessed the effect of different tidal volumes (V_T), oxygen and endotoxin on cytokine/chemokine production in lungs of newborn rat that were ventilated for eight hours. We demonstrated that low-tidal volume ventilation resulted in a mild inflammatory response. Gene expression of pro-inflammatory cytokines/chemokines CXCL-2 and IL-6 was significantly increased. A further increase was observed with increasing V_T and ventilation with 50% oxygen, but not after pre-exposure to endotoxin. The increase in mRNA expression was accompanied by an increase of IL-1β and/or IL-6 protein in the bronchoalveolar lavage fluid (BALF) after high-V_T ventilation and ventilation with oxygen and ventilation after exposure to endotoxin. Elevated concentrations of these cytokines/chemokines in amniotic fluid and BALF have been associated with BPD. The underlying mechanisms by which stretching of a developing lung may disturb lung development are described in the next chapters.

Chapter 4 reviews apoptotic cell death in lung development and BPD. Insight in lung development is crucial to study the pathogenesis of BPD. Throughout each stage of lung development apoptosis is important for remodelling of lung tissue. Apoptotic activity is genetically determined but can also be triggered by stretch/mechanical ventilation, hyperoxia and inflammation. Hence, it is not surprising that dysfunctional apoptotic mechanisms are believed to be important in the development of BPD. Recent studies provide strong evidence for Fas/FasL dependent epithelial apoptosis. Candidates for activation of apoptotic pathways by oxygen are: pro-apoptotic Bax protein, cell cycle regulator p53 and cytokine TGF-β. Neutrophils play a critical role in inflammation-induced apoptosis. They release pro-apoptotic mediators but also have a prolonged half-life due to diminished apoptosis with triggers an ongoing inflammatory response.

In **Chapter 5** we investigated the signalling mechanisms of ventilation (stretch)-induced apoptosis in newborn rat lung. Mechanical ventilation of newborn rats for 24 hours with moderate V_T induced apoptosis of alveolar type II cells. This coincided with activation of the Fas/FasL system. Involvement of the Fas/FasL system was confirmed by continuous cyclic stretching of fetal lung epithelial cells. Not only increased stretch expression of FasL by the cells, but neutralizing FasL antibodies abrogated this increase. Together with increased expression of FasL, we observed increased expression of cleaved caspase-3, -7 and -8, but not of caspase-9. These data indicate activation of the extrinsic death pathway *via* the FasL/Fas system by prolonged mechanical ventilation, resulting in apoptosis of alveolar type II cells.

Chapter 6 focuses on the effect of prolonged mechanical ventilation on lung cell proliferation. A decrease in the number of proliferating lung cells was observed in new born rats with increase of duration of mechanical ventilation. After ventilating for 24 hours lung cell proliferation came to a halt in the G1 phase of the cell cycle. This was associated with an arrest in alveolar development as demonstrated by a decrease in secondary crests and larger alveoli. For transition of the cell cycle from G1 into S phase it is crucial that cyclin-dependent kinases (Cdk) 4/6 form a complex with cyclin D as well as Cdk2 with cyclin E. We found increased expression of Cdk inhibitor $p27^{Kip1}$ and $p57^{Kip2}$ what prevents binding of the Cdks to its Cyclin partner. These findings were confirmed *in vitro* by continuous cyclic stretching of fetal lung epithelial cells. All together, these experiments revealed a new mechanism by which mechanical ventilation inhibits cell proliferation.

The aim of the experiments described in **Chapter 7** was to investigate the influence of (prolonged) mechanical ventilation, inflammation and hyperoxia on the expression of genes regulating the formation of alveoli and blood vessels in lungs of newborn rats. The current view is that the development of the blood vessels in the lung passively follows that of the airways. However, increasing evidence suggests that lung angiogenesis actively promotes alveolar growth during development via the secretion of angiogenic growth factors. A better understanding of both developmental processes may have potential for the treatment of BPD and other lung diseases characterized by disrupted alveolarization. Our findings suggest an imbalance between the expression of Fibulin5 and tropoelastin due to mechanical ventilation resulting in a dysregulated elastin assembly. A systemic inflammation promoted this ventilation-induced imbalance in elastogenic gene expression. A disturbance in angiogenesis was initiated by altered PDGF and VGF receptor gene expression. These changes in mRNA expression of angiogenic receptors was only observed after moderate tidal volume ventilation, not after ventilation with low tidal volumes.

Chapter 10

Appendices

NEDERLANDSE SAMENVATTING

In **hoofdstuk 1** – de algemene introductie - worden de huidige inzichten ten aanzien van BPD in relatie tot longontwikkeling en longschade bediscussieerd. De onderliggende gedachte over de ontstaanswijze van BPD is dat de long van te vroeg geboren kinderen structureel niet volgroeid is. Iedere gebeurtenis, welke voor of na de geboorte optreedt gedurende deze periode, kan het ontwikkelingsprogramma van de onvolgroeide long nadelig beïnvloeden. De pathologische bevindingen, welke gezien worden bij BPD, zijn het gevolg van een complex samenspel tussen trauma's voor de geboorte (infectie, chorioamnionitis), tekortkomingen van de onrijpheid van de long (onvoldoende surfactant, minder ontwikkeld anti-oxidant systeem) en beschadigingen ten gevolge van levensreddende behandelingen zoals beademing en extra zuurstof toediening. Gezamenlijk leidt dit tot een chronische ontsteking van de long, welke het ontstaan van longblaasjes en longvaten schaden. Beide processen zijn essentieel voor de normale ontwikkeling van de long en spelen zonder twijfel een rol in de ontstaanswijze van BPD.

De redenen waarom de studies werden uitgevoerd die beschreven zijn in dit proefschrift worden genoemd in **hoofdstuk 2**.

In **hoofdstuk 3** is het effect van verschillende teugvolumina (V_T), zuurstof en endotoxinen op de cytokine/chemokine produktie bepaald in de long van pasgeboren ratten, die gedurende acht uur werden beademd. Het beademen met een klein teugvolume resulteerde in een geringe ontstekingsreactie. De gen expressies van pro-inflammatoire cytokines/chemokines CXCL-2 en IL-6 waren significant verhoogd. Een verdere toename werd vastgesteld met toename van het teugvolume en beademing met 50% zuurstof, maar niet na behandeling met endotoxine. De toename van mRNA expressie ging samen met een toegenomen hoeveelheid van het eiwit IL-1β en/of IL-6 in de bronchoalveolaire lavage vloeistof (BALF) na het beademen met een groot teugvolume. Dit vond ook plaats bij het beademen met zuurstof of bij behandeling met endotoxine. Toegenomen concentraties van deze cytokines/chemokines (IL-1β en/of IL-6) in amnion vloeistof en BALF zijn geassocieerd met BPD. De onderliggende mechanismen waardoor het oprekken van de zich ontwikkelende long kunnen leiden tot een verstoorde longontwikkeling, worden in de volgende hoofdstukken beschreven.

In **hoofdstuk 4** wordt een overzicht gegeven van de rol van apoptotische cel dood gedurende de normale longontwikkeling en de ontwikkeling van BPD. Kennis van de longontwikkeling is essentieel voor het bestuderen van het ontstaan van BPD. Gedurende ieder ontwikkelingsstadium van de long is apoptosis belangrijk. Apoptotische

activiteit is genetisch vastgelegd, maar kan ook gestimuleerd worden door rek/beademing, hyperoxie en ontsteking. Het is dus niet verrassend dat wordt verondersteld dat verstoorde apoptotische processen van belang zijn voor het ontstaan van BPD. Recente studies tonen overtuigend aan dat apoptose van de epitheliale cellen afhankelijk is van het Fas/FasL systeem. Kandidaten voor activatie van apoptose ten gevolge van zuurstof zijn: het pro-apoptotische eiwit Bax, cel cyclus regulator p53 en cytokine TGF-β. Witte bloedlichaampjes spelen een kritische rol in door ontsteking veroorzaakte apoptosis. Uit de witte bloedlichaampjes komen pro-apoptotische mediatoren vrij, maar ook hebben ze een verlengde levensduur ten gevolge van verminderde apoptosis. Dit geeft aanleiding tot een aanhoudende ontsteking.

In **hoofdstuk 5** hebben we mechanismen onderzocht hoe beademing kan leiden tot apoptose in de long van pasgeboren ratten. Beademing van pasgeboren ratten gedurende 24 uur met middelmatige teugvolumina veroorzaakte apoptosis van de alveolaire type II cellen. Dit ging gepaard met activatie van het Fas/FasL systeem. Betrokkenheid van dit systeem werd bevestigd door het continu cyclisch rekken van foetale epitheliale longcellen. Niet alleen versterkte het rekken de expressie van FasL door deze cellen, maar met het toedienen van neutraliserende FasL antilichamen was de apoptosis verminderd. Gelijktijdig met de toegenomen expressie van FasL, was ook de expressie van 'cleaved caspase-3, -7 en -8' toegenomen, maar niet die van caspase-9. Deze data tonen aan dat beademing de 'extrinsic death pathway' via het FasL/Fas systeem activeert met apoptose van de alveolaire type II cellen als gevolg.

Hoofdstuk 6 belicht het effect van langdurige beademing op het delen (proliferatie) van longcellen. Een afname in het aantal proliferende longcellen van pasgeboren ratten werd waargenomen met toename van de duur van de beademing. Na een beademingsperiode van 24 uur stopte de proliferatie van de longcellen in de G1 fase van de celdeling. Dit ging samen met een remming van de ontwikkeling van de longblaasjes: het aantal 'secundaire crests' was afgenomen en de longblaasjes waren groter. Voor transitie van de cel cyclus van de G1 naar de S fase is het van belang dat cyclineafhankelijke kinases' (Cdk)4/6 een complex vormen met cycline D en Cdk2 met cycline E. Wij toonden een toegenomen expressie aan van de eiwitten $p27^{Kip1}$ and $p57^{Kip2}$. Deze eiwitten voorkomen binding van Cdks met de bijbehorende cycline. Deze bevindingen werden bevestigd *in vitro* door het continu cyclisch rekken van foetale epitheliale longcellen. Door deze experimenten hebben we een nieuw mechanisme aangetoond hoe beademing de deling van longcellen remt.

Wat is de invloed van (langdurige) beademing, ontsteking en hyperoxie op de expressie van genen die de ontwikkeling van longblaasjes en bloedvaten in de longen

van pasgeboren ratten reguleren? Dit wordt beschreven in hoofdstuk 7. Het huidige inzicht is dat de ontwikkeling van de bloedvaten in de long de ontwikkeling van de longblaasjes passief volgt. Er is zijn echter toenemende aanwijzingen dat de bloedvat vorming in de long de groei van longblaasjes aktief stimuleert. Een beter inzicht in beide ontwikkelingsprocessen kan mogelijk leiden tot de behandeling van BPD en andere longziekten die worden gekenmerkt door door een gestoorde ontwikkeling van de longblaasjes. Onze bevindingen suggereren dat beademing de balans tussen de expressie van fibulin5 en tropoelastin verstoort met als gevolg een verstoorde samenstelling van de elastine vezels. Een systeem infectie stimuleert deze door de beademing veroorzaakte imbalans via genen die betrokken zijn bij de vorming van de elastine vezels. Een verstoring van bloedvat vorming werd veroorzaakt door een veranderde expressie van PDGF en VGF receptor genen. Deze veranderingen in mRNA expressie werd alleen waargenomen na het beademen met middelmatige teugvolumina, niet na beademen met kleine teugvolumina.

Tot slot wordt in de discussie (hoofdstuk 8) alle bevinding in perspectief geplaatst en worden aanbevelingen gedaan voor verder onderzoek.

CURRICULUM VITAE

André Kroon was born as 'Amsterdammer' on the 21st of December 1964. He went to secondary school at the S.G. Sint Nicolaas Lyceum in Amsterdam from 1977 to 1983. After graduating, he started his medical training at the Medical Faculty of The Vrije University of Amsterdam in 1984 and received his medical degree cum laude in 1990. He joined the military service and worked as first lieutenant physician at the ENT department of the Central Military Hospital in Utrecht from 1991 to 1992. There after a long career started as paediatric resident-not-in-training. First at the Sint Lucas Hospital in Amsterdam from 1992 to 1994, next at the Academic Medical Centre in Amsterdam from 1994 to 1996 and finally in the Erasmus MC-Sophia in 1996, where he became pediatric resident-in-training in 1997 (Prof. Dr. H.J. Neijens). The non-academic part of the training was followed in the Zuiderziekenhuis in Rotterdam (dr. R. Sukhai). In 2001 he started his fellowship Neonatology in the Erasmus MC-Sophia under supervision of dr. J.N. van den Anker. He finished it in 2003 under supervision of Prof. dr. L.J.I. Zimmerman, who made him enthusiastic for lung research. Since then he is staffmember of the Department of Neonatology, Erasmus MC-Sophia. In 2005 he started his PhD thesis, when he went for the first time to Toronto. He started a research fellowship in the laboratory of the Department of Lung Biology at the Hospital for Sick Children under guidance of Prof. dr. M. Post. In 2009 he went for another year as research fellow to Toronto.

André became 'Rotterdammer' in 2011. He lives happily together with George Damhuis.

LIST OF PUBLICATIONS

Kroon AA, Heij JMH, Kuijper WA, Veerman AJP, v.d. Baan S. Function and morphology of respiratory cilia in situs inversus. Clinical Otolaryngology. 1991 Jun; 16(3):294-7.

Kroon AA, Schutte MF, Swartjes JM. Circulatiestilstand ten gevolge van een te hoge dosis magnesiumsulfaat bij een zwangere met een eclamptisch insult. Nederlands Tijdschrift voor Obstetrie en Gynaecology. 1991 May; 104:130-3.

Kroon AA, Smit BJ, Hennekam RCM. A patient with the OEIS complex. Pediatric Clinics Amsterdam. 1995 Jan; 6(1):21-2.

Kroon AA, Ramaker C, Keessen M, Derkx HHF, Büller HA, Tamimiau JAJM.
Ileïtis terminalis op de kinderleeftijd, M. Crohn of gastrointestinale tuberculose? Nederlands Tijdschrift voor Geneeskunde 1995. Oct; 139(40):2017-20.

Kroon AA, Smit BJ, Hennekam RCM. An unusual presentationn of trisomy 18. Pediatric Clinics Amsterdam. 1995 May; 6(3):9-10.

Kroon AA, v. Deutekom H, Derkx HHF. Abdominale tuberculose bij kinderen. Nederlands Tijdschrift voor kindergeneeskunde. 1996 Dec; 64(6):276-81.

Kroon AA, Smit BJ, Hennekam RCM, Barth PG. Lissenchephaly with extreme cerebral and cerebellar hypoplasia. Neuropediatrics. 1996 Oct; 27(5):273-6.

ten Berge M, Brinkhorst G, Kroon AA, de Jongste JC. Dnase treatment primary ciliary-dyskinesia – assesment by nocturnal pulse oximetry. Pediatric Pulmomology. 1999 Jan; 27(1):59-61.

Dik WA, van Kaam AH, Dekker T, Naber BA, Janssen DJ, Kroon AA, Zimmermann LJ, Versnel MA, Lutter R. Early increased levels of matrix metalloproteinase-9 in neonates recovering from respiratory distress syndrome. Biol Neonate. 2006 Sep; 89(1):6-14.

van der Sluis IM, Boot AM, Vernooij M, Meradji M, Kroon AA. Idiopathic infantile arterial calcification: clinical presentation, therapy and long-term follow up. Eur J Pediatr. 2006 Sep; 165(9):590-3.

Kroon AA, Wang J, Huang Z, Cao L, Kuliszewski M, Post M. Inflammatory response to oxygen and endotoxin in newborn rat ventilated with low tidal volume. Pediatr Res. 2010 Jul; 68(1):63-9.

van Kaam AH, Rimensberger PC, Borensztajn D, De Jaegere AP; Neovent Study Group. Ventilation practices in the neonatal intensive care unit: a cross-sectional study. J Pediatr. 2010 Nov; 157(5):767-71.e1-3.

Kroon AA, Wang J, Kavanagh B, Huang Z, Kuliszewski M, van Goudoever JB, Post M. Prolonged mechanical ventilation induces cell cycle arrest in newborn rat lung. PLoS One. 2011 Feb; 6(2):e16910.

Kroon AA, Post M. Apoptotic Cell Death in Bronchopulmonary Dysplasia. Current Pediatric Reviews. 2011 Nov; 7(4):285-292.

PhD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: A.A. Kroon	PhD period: 2005-2011
Erasmus MC Department: Neonatology	Promotors: Prof. Dr. J.B. van Goudoever
Research School: Erasmus MC	Prof. Dr. M. Post

1. PhD training

	Year	Workload (hours)
General courses		
- Biological, Chemical and Radiation Safety Seminars Research Integrity	2005/2009	48
- Lab Animal Services General Orientation	2005	4
- Lab Animal Services Handling	2005	4
- Lab Animal Services Gas Anesthesia	2005	4
- Lab Animal Rodent Survival Surgery	2005	4
- Introduction to Data-analysis	2007	35
Seminars and workshops		
- Research meetings 'Moeder en Kind' Center	2006-2011	20
- Improving Mechanical Ventilation in Neonates, Gross Doelln	2008	16
- Research meetings 'Lung Biology Research, Physiology and Experimental Medicine Program'	2005/2009	150
Presentations		
- Prolonged mecanical ventilation alters gene expression in newborn rats (SPR, Washington)	2006	30
- Influence of mechanical stretch on lung development (Research in progress meeting, Toronto)	2006	30
	2006	30
- Breathless in Toronto (Grand Round, Erasmus MC-Sophia, Rotterdam)	2007	30
- Schade ten tijde van de longontwikkeling (Onderzoeksdag, Erasmus MC-Sophia, Rotterdam)	2008	60
- Oxygen and Inflammation alter alveolarisation in newborn rats ventilated with low tidal volumes (SPR, Honolulu)	2009	60
- Ins and Outs of Bloodgas analysis (Boerhaave cursus, Leiden)		
- Oxygen and Inflammation alter alveolarisation in newborn rats ventilated with low tidal volumes (ESPR, Hamburg)	2009	60
- Prolonged ventilation causes $p27^{KIP1}$ induced cell cycle arrest in newborn rat lung (ESPR, Kopenhagen)	2010	30
(Inter)national conferences		
- Hot Topics in Neonatology, Denver	2007	28
- Nederlandse Nascholing Neonatologie, Tegelen	2006/2010	56
- SPR, Denver	2011	28
2. Teaching		
- Introduction training internship pediatrics, Rotterdam	2006-2011	150
- NICU nurses education, Rotterdam	2006-2011	50
- Medical training pediatric residents, Rotterdam	2006-2011	50

LIST OF ABBREVIATIONS

BALF	bronchoalveolar lavage fluid
BPD	bronchopulmonary dysplasia
BrdU	5-bromo-2-desoxyuridine
CDK	cyclin dependent kinase
ECM	extra cellular matrix
Egr	early growth response
FBM	fetal breathing movements
Fib5	fibulin5
FLF	fetal lung fibroblast
FLDE	fetal lung epithelial cel
GA	gestational age
HFOV	high frequency oscillatory ventilation
HV_T	high tidal volume
IL	interleukin
LOX	lysyl oxidase
LPS	lipopolysaccharide
LV_T	low tidal volume
MPO	myeloperoxidase
MV	mechanical ventilation
MV_T	moderate tidal volume
NV	non-ventilated
PDGF	platelet-derived growth factor
PECAM	platelet endothelial cell adhesion molecules
PEEP	positive end expiratory pressure
RDS	respiratory distress sydrome
ROS	reactive oxygen species
TN-C	tenascin-C
TNF	tumor necrosis factor
TO	tracheal occlusion
VEGF	vascular endothelial growth factor
VILI	ventilator-induced lung injury
V_T	tidal volume