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REVIEW

Apoptosis in Lung Development and Neonatal Lung Injury

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ABSTRACT

A healthy organism maintains an integrated balance between proliferating, differentiating, and dying cells. Some cells are irreplaceable, some cells complete their functions and are then sacrificed, and some cells live a finite lifetime, to be replaced by another generation. Apoptosis is the last phase of a cell's destiny and a distinct form of programmed cell death. It is characterized by loss of cell function and rapid morphological changes, culminating in cell death without inflammation. Apoptosis has been found to play an important role in the normal regulation of organogenesis and morphogenesis during development. Apoptosis is a fundamental feature in the development of many tissue systems, including the immune and nervous systems, as well as in the development of the kidneys and heart. The significance of apoptosis in lung development has been largely overlooked. Physical forces during development may play a role in directing apoptosis in remodeling the lung. This review summarizes current knowledge regarding apoptosis during lung development, with a particular emphasis on the potential role of mechanpotransduction as a stimulus for apoptosis. **(*Pediatr Res* 55: 183–189, 2004)**

Abbreviations
AEC, alveolar epithelial cell
TGF-β1, transforming growth factor-β1
FBM, fetal breathing movements
Apaf-1, apoptotic-protease-activating factor-1
BPD, bronchopulmonary dysplasia
RDS, respiratory distress syndrome

Apoptosis, derived from the Greek word for a natural process of leaves falling from trees, is a distinct form of programmed cell death characterized by loss of cell function and rapid morphologic changes, culminating in cell death without inflammation. Apoptosis was first introduced into modern scientific writing by Kerr *et al.* (1) and has since been found to play an important role in the normal regulation of organogenesis and morphogenesis during development. Apoptosis plays a vital role in the development of many tissue systems, including the immune (2) and nervous systems (3, 4), as well as in the development of the kidneys (5) and heart (6). In view of the well-established role of apoptosis in developmental modeling processes, it seems plausible that apoptosis is important for lung development. This review focuses on the importance of mechanotransduction during lung development and its potential role as a stimulus for apoptosis. However, other equally important developmental regulators of lung growth and differentiation, including oxygen tension, glucocorticoids, and other hormonal factors, also need to be considered as potential stimulators of apoptosis.

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APOPTOSIS AND LUNG DEVELOPMENT

To provide gas exchange efficiently, the lung undergoes dramatic tissue growth and remodeling both *in utero* and after birth. Lung development comprises six different stages (Table 1). During the embryonic stage, the lung primordium appears as a ventral diverticulum of the foregut. It separates from the prospective esophagus and elongates caudally. The resulting bud branches for the first time and gives rise to the main bronchi of the left and right lungs. These bronchi divide dichotomously and invade the surrounding mesenchyme, forming the future airways. Looking like a primitive gland, the developing lung enters the pseudoglandular stage. During this period, most of the branching morphogenesis takes place (7). In the subsequent canalicular stage the bronchial tree is established, vascularization increases rapidly, respiratory bronchioli

Table 1. *Lung development and the role of apoptosis*

Stage	Major events	Role of apoptosis	Reference
Embryonic	Lung anlage appears Branching morphogenesis starts with extensive proliferation of epithelial and mesenchymal cells Few pulmonary vascular connections established	Apoptosis in mesenchyme around branch points and regions of new lung bud formation No epithelial apoptosis	14
Pseudoglandular	The bronchial airway tree is established by repeated dichotomous branching Airways are lined with thick epithelium while neuroendocrine, ciliated, globular, and Clara cells start to differentiate	Apoptosis of interstitial tissue contributes to mesenchymal involution No epithelial cell apoptosis	9–11
Canalicular	Respiratory bronchioli appear ↓ interstitial tissue → airway widening Differentiation of type II into type I cells Rapid increase of vascular network	Apoptosis of interstitial tissue contributes to mesenchymal involution and thinning of the alveolar septa ↑ apoptosis of epithelial cells as cell proliferation ↓	9–13
Saccular	Terminal airways widen to form saccules Thinning of interstitium between airspaces Vascular network expands	↑ epithelial cell apoptosis	9–13
Alveolar	Extensive alveolar septation; ↑ in alveoli Further thinning of epithelial-endothelial barrier	Transient ↑ ⇈ in apoptosis at birth Continuous proliferation of epithelial cells	20,21
Microvascular	Double capillary layer in alveolar septa is reduced to a single layer	Final ↑ in apoptosis, to remove excess cells	20,21

↑, increase, ↓, decrease.

appear, interstitial tissue decreases, and the epithelial barrier thins out as the capillaries begin to lie in close apposition to the epithelium, forming the eventual air–blood barrier (7). By the end of this stage, airway branching is complete and the distal cuboidal epithelium begins to differentiate into alveolar epithelial type II cells (AEC2) and alveolar epithelial type I cells (AEC1), responsible for surfactant production and gas exchange, respectively (7). During the saccular stage of lung development, just before birth, epithelial and air space compartments expand, resulting in terminal clusters of widened spaces called "saccules." The capillary network surrounding the saccules condenses. As a result, the septa now contain two sheets of capillary layers separated by a core of connective tissue. During the period of alveolarization, the gas exchange surface is greatly enhanced by the establishment of secondary septa stemming from the existing primary septa (7). The double capillary layer of the immature alveolar septa is reduced to a single capillary layer during the final developmental phase of microvascular maturation (8).

Apoptotic activity has been observed during all stages of lung development, suggesting its important role during this highly orchestrated process (Table 1). In 1998, two independent studies (9, 10) reported the occurrence of apoptosis in fetal lung development. *In vivo* detection of apoptotic cells is very difficult to assess by morphologic methods because tissue systems have very efficient and rapid clearance mechanisms. However, using three well-established methods to detect apoptosis (light and electron microscopy, the nucleosomal ladder pattern of DNA digestion, and the detection of apoptotic cells *in situ* by the terminal deoxynucleotidyl transferase-mediated nick-end labeling method), a shift in apoptosis was observed from the mesenchymal tissue layer during the earlier stages of development (9) to both the epithelial and mesenchymal tissue layers during the canalicular stage of development and onward (10, 11). Increasing AEC2 apoptosis coincided with a decrease in cell proliferation, implicating epithelial apoptosis as a significant contributor of lung remodeling during late gestational development (12, 13). Throughout the embryonic stage of lung development, apoptosis was almost exclusively found in the peripheral mesenchyme in regions of new bud formation or in the mesenchyme underlying branch points that are the site of extensive epithelial branching morphogenesis and remodeling of interstitial tissue, allowing room for outgrowth of the lung bud (10, 14). It is interesting that mesenchymal cells undergoing apoptosis were intermingled with proliferating mesenchymal cells (14), suggesting that a coordination of these two processes is important in the cell dynamics associated with bronchial branching. Apoptosis during development may be mediated by proapoptotic factors, such as transforming growth factor-β1 (TGF-β1) (15), and antiapoptotic factors, such as IGF-1 (16), nitric oxide (17, 18), and secreted apoptosis-related proteins (19), that all are found in the alveolar environment.

After birth, apoptosis emerges as an important process after extensive proliferation and subsequent transformation of primary saccules into functional alveoli (20, 21). In rat, during the first 2 wk of life, with distension of alveolar air spaces, fibroblasts and AEC2 are produced in greater numbers than are actually needed at the end of alveolarization (21, 22). Excess of AEC2 is important because they serve as the putative stem cell for AEC1, which line most of the alveolar surface and are critical for the maturation of the air–blood barrier during microvascularization (23). Also, because AEC2 are the producers of surfactant, an excess production ensures sufficiently available surfactant at and just after birth (7). After alveolarization, excess AEC2 decrease in number by differentiating into AEC1 and by apoptosis (20). This wave of apoptosis takes place during the final phase of microvascular maturation, resulting in significant thinning of the thick alveolar septa. There is also a sharp increase in apoptotic activity of pulmo-

nary rat fibroblasts (20, 21). A large proportion of AEC2 cells stain positive for apoptosis during the postnatal period but do not exhibit typical nuclear fragmentation of apoptotic bodies evident in fibroblast cells, suggesting that AEC2 may be cleared by alveolar macrophages in early stages of the apoptotic process (20). Together, these findings support a role for apoptosis in establishing a proper gas exchange surface both *in utero* and after birth.

BIOPHYSICAL STIMULI OF APOPTOSIS

In utero, the lung is continuously exposed to a variety of physical forces, including fetal breathing movements (FBM) and tonic distension (24, 25). FBM are the characteristic intermittent breathing movements that mechanically stimulate the lung and facilitate fluid pressure changes. This active secretion of fluid by the fetal epithelium sustains a constant pulmonary pressure. FBM have been observed in the fetus starting in the first trimester and increase until term (25). During lung development, as mechanical forces increase *via* liquid secretion and FBM, proliferation of lung cells increases as well, peaking at the canalicular stage of development and decreasing thereafter (26, 27). While proliferation levels are decreasing, apoptotic activity increases (10, 11). The same pattern is observed postnatally, during alveolarization, when the lung is experiencing high levels of airspace distension as the infant is breathing on its own. Distension of alveolar air spaces results in proliferation of fibroblast and epithelial cells, followed by apoptosis of these cells (20, 21).

Many studies have confirmed cell deformation as the mechanical stimulus that promotes growth of the fetal lung. Stretch induces DNA synthesis and cell division in fetal lung organotypic cultures (28–30), fetal lung fibroblasts (29, 31), and fetal AEC2 cells (32). Apoptosis was not described in these proliferation studies because it was not specifically looked at and it is a short-lived event. Apoptosis is a dynamic process that is rapidly completed, often in <1 h (33), whereas cell proliferation occurs at a much slower rate (between 12 and 24 h) in the developing lung (34). A role for stretch-induced apoptosis in lung development was first suggested by De Paepe *et al.* (11), who showed that depletion of AEC2 after tracheal ligation in fetal rabbits was at least in part due to an increase in apoptosis. Similarly, 20% radial elongation stimulated apoptotic activity in primary adult AEC2 (35). However, these studies performed tissue distension under conditions greater than those considered "physiologic" during gestation. Lung fibroblasts from the canalicular stage of development, cultured in a mechanically active environment simulating FBM, showed increased levels of apoptosis, contrary to cells from the pseudoglandular and saccular stages (36). Therefore, mechanical forces seem to stimulate apoptosis of fetal lung fibroblast cells in a time-specific manner and may be involved in the thinning of the periacinar mesenchyme during late fetal lung development. However, it should be mentioned that stretching of isolated cell populations eliminates the effect of epithelial-mesenchymal signaling that is vital for proper lung development and that one should be careful in extrapolating *in vitro* data to *in vivo*.

Thus, it seems that early in gestation, physical stimuli for proliferation and apoptosis come from a forward-pushing movement of the growing lung bud as well as the forward pushing of the liquid fluid inside the airways (37). The living cells filter the same chemical input from local mechanical deformations and decide to proliferate or die (38), allowing apoptotic and proliferating cells to coexist in the same mesenchymal environment. In addition to these physical stimuli, local growth factor signaling (*e.g.* fibroblast growth factor-10, sonic hedgehog and bone morphogenetic factor-4) determines the final fate of the cell (39). The tandem of apoptosis and proliferation, early in gestation, is necessary to support a growing lung and to create room needed for growth. Epithelial apoptosis becomes important in the later part of gestation with alveolar growth and seems to be stimulated *in utero* by increasing FBM and postnatally by airway distension. In addition, FBM may control ongoing mesenchymal condensation *via* apoptosis.

MECHANOTRANSDUCTION AND APOPTOSIS

The strong association between the degree of airspace distension and apoptotic activity suggests the existence of a mechanotransduction process through which airspace stretch triggers an apoptotic pathway in epithelial and mesenchymal cells. How distension interacts to produce normal lung growth and by what mechanism this physical stimulus induces proliferation and/or apoptosis of lung cells remains unknown. One possible explanation is through the effect of stretch on cell cycle control. Cells undergoing mitosis and apoptosis share common pathways with the same morphologic and biochemical changes, including rounding of the cell, release of substrate attachments, decrease in cell volume, chromatin condensation, and expression of cell cycle genes (40, 41). Apoptosis and proliferation are linked by cell cycle regulators (41), and stimuli such as distension affect both cell proliferation and death. Initially, in the developing lung, mechanical distension may encourage cells to enter the S phase of the cell cycle, leading to increased DNA synthesis and increased levels of proliferation. However, during the later stages of lung development, further increases in distension or a feedback mechanism may interfere with the normal cell cycle, driving cells into the Gap 0 (G_0)/G_1 phase and eventually to cell death. Fetal AEC2 express several key cyclins and cyclin-dependent kinases, unlike adult AEC2 (42). In rat fetal fibroblasts, isolated at the canalicular stage of lung development and subjected to stretch, cell cycle progression was inhibited and greater numbers of cells were found in the G_0/G_1 phase, whereas fewer cells were found in the S phase (36).

Cell–cell and cell–matrix contacts are vital for proper lung development and may play an important role in mechanosensing and -transduction. Cells adhere to neighboring cells and to the extracellular matrix *via* transmembrane receptors of the cadherin and integrin families, respectively. Connections between integrin-rich focal adhesion sites, cytoskeletal filaments, and nuclear scaffolds provide a discrete path that mechanically couples the cytoplasmic portion of integrins to the actin cytoskeleton allowing for mechanical signal transfer (43–45).

Activation of c-src, a member of the Src family kinases, is a common biochemical response to mechanical stimulation in many cells (Fig. 1). Upon stretch in fetal lung cells, actin filament-associated protein, distributed along the actin filaments, recruits and activates c-src (46). Uni-axial cyclic stretch induces c-src activation and translocation in human endothelial cells *via* stretch-activated channel activation (47). Furthermore, fibroblast shape is considerably altered in response to stretch, and the signaling mechanism responsible may be *via* activation of c-src (48, 49). Pressure overload in the rat myocardium induced an increase in focal adhesion kinase, which may act in collaboration with c-src, in turn activating signaling pathways involved in multiple cellular processes (48, 50). Whether c-src is involved in apoptosis remains to be investigated (Fig. 1). Other signaling pathways involved in apoptosis include the Ras signaling pathway, the mitogen-activated protein kinase pathway, and the phosphatidylinositol 3-kinase pathway, which mediate downstream effects on transcription factors and gene expression (51, 52). Furthermore, cells may sense their degree of extension or compression and thus monitor local changes in cell crowding or extracellular matrix compliance and thereby couple changes accordingly. Thus, information from mechanical signals resulting in changes in integrin signaling together with changes in cell shape may be organized by focal adhesions that integrate and orient much of the signal transduction machinery of the cell, resulting in apoptosis or proliferation (38, 53).

APOPTOSIS SIGNALING PATHWAY IN FETAL LUNG

The signaling pathway that mediates apoptosis of fetal epithelial cells is not clear but most likely involves caspase-dependent pathways (Fig. 2). One potential candidate is the Fas–Fas ligand (FasL) system, which may play a role in controlling epithelial cell homeostasis (Fig. 1). Fas (CD95/APO1), a member of the tumor necrosis factor-α receptor family, binds FasL, thereby inducing trimerization of Fas in the target cell membrane. FasL expression has been observed in the adult murine lung and has a spatiotemporal pattern of expression in the developing rabbit lung, where it is highly expressed in AEC2 (54). The time-specific up-regulation of FasL mRNA and protein during development is highly suggestive of a tightly up-regulated transcriptional or posttranscriptional regulation of the FasL gene. Fas is continuously expressed in the fetal lung, and apical Fas receptors are present on bronchiolar Clara cells and AEC2 (54). The up-regulation of FasL expression corresponds with increasing AEC2 apoptosis and maximal airway distension (54), implicating Fas-FasL as a mediator of late-gestational apoptosis, mediated by stretch. The Fas-FasL pathway is pivotal in activating upstream initiator caspases 8 and 10, in turn activating effector caspase 3, culminating in apoptosis. Procaspase-8 mRNA expression peaks at day 7 of embryonic murine life and remains fairly high onward (55). A homozygous disruption of the mouse caspase 8 gene has been found to be lethal *in utero* (56), and these mice had impaired heart muscle development and extensive eryth-

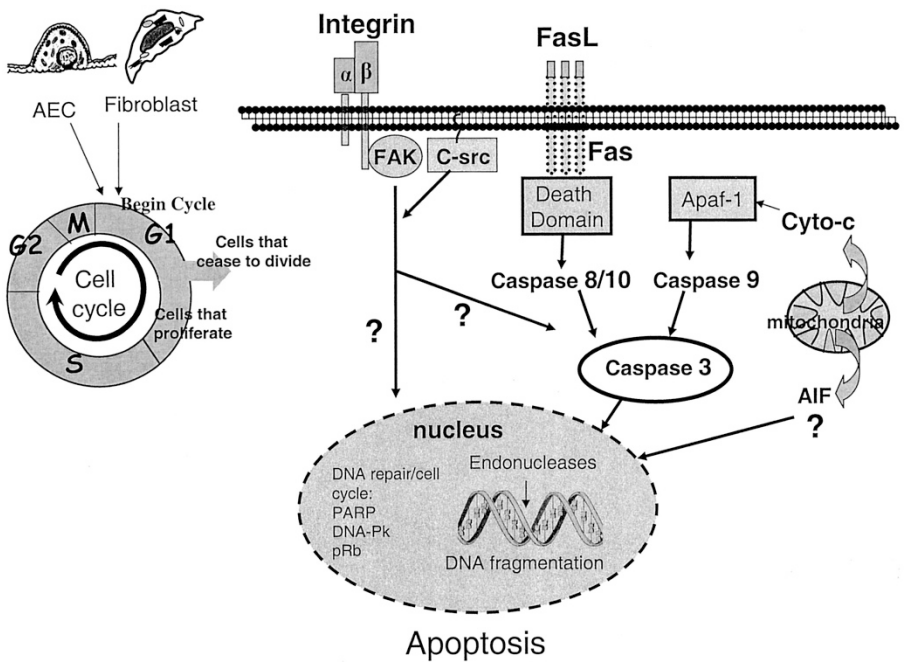

Figure 1. During the G_1 phase of the cell cycle, fetal epithelial and fibroblast cells may decide to proliferate or die. In the fetal lung, apoptosis may be orchestrated by cell–matrix interactions (*e.g.* integrin/focal adhesion kinase/c-src), caspase-dependent pathways (*e.g.* caspase 8/10 or 9, and 3), or caspase-independent pathways (*e.g.* apoptosis-inducing factor). All pathways may coordinate the execution phase of apoptosis by cleaving many key proteins such as nuclear enzyme poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-pk), retinoblastoma gene (pRb), and degradation of chromosomal DNA.

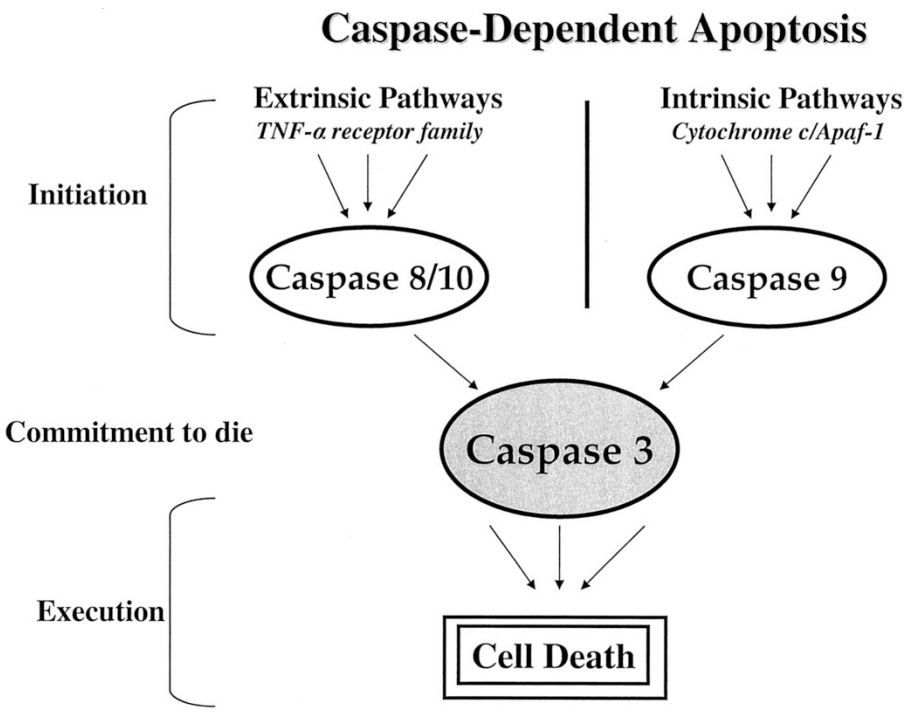

Figure 2. Caspase 3 is the primary executioner caspase and may be activated *via* two main routes during lung development: *1*) intrinsic mitochondrial-cytochrome *c*–dependent pathway requiring caspase 9 or *2*) an extrinsic pathway initiated by the Fas/FasL system triggering caspase 8/10 activation, in turn activating caspase 3.

rocytosis in the liver and lungs, perhaps caused by a defect in angiogenesis. However, overall, these mutant mice displayed normal lung development, which questions the role of caspase 8 during fetal lung development. As mentioned earlier, another caspase activated by the Fas-FasL system is caspase 10. Although caspase-10 is highly expressed in the fetal lung, its exact role in apoptosis remains to be established (57).

It is likely that multiple proapoptotic signaling pathways are involved during development because FasL up-regulation was short lived in fetal rabbit lungs, even though AEC2 apoptosis continued (54). One candidate may be the intrinsic mitochondrial-cytochrome *c*–dependent pathway requiring caspase 9 (Figs. 1 and 2). Cytochrome *c* seems to act by forming multimeric complexes with apoptotic-protease-activating factor-1 (Apaf-1), which in turn activates caspase 9 (58). Apaf-1 expression is very high in fetal lung (59), correlating with high levels of apoptosis. However, even though both caspase 9– and Apaf-1–deficient mice die around day 16.5 of development, the lung seems unaffected and remarkably normal (60).

The mitochondrial-cytochrome *c*–dependent pathway may be important in mediating apoptosis in fetal lung mesenchymal cells, which do not express Fas or FasL (36, 54). Apoptotic activity is relatively high in these cells, and stretch activates caspase 3 activity (36). However, homozygous mice deficient for caspase 3 die during the first weeks after birth with no noticeable lung abnormalities (61, 62), suggesting that caspase-independent pathways may also be involved. One possible candidate involved in the apoptotic mesenchymal reorganization is apoptosis-inducing factor (Fig. 1), which is localized to the mitochondria and released in response to death (63). Apoptosis-inducing factor–dependent cell death is crucial during early mouse morphogenesis, but its role in lung development is not yet known.

APOPTOSIS IN NEONATAL LUNG INJURY

Bronchopulmonary dysplasia (BPD) describes the postnatal lung pathology caused by a combination of mechanical ventilation and oxygen in preterm infants who have neonatal respiratory distress syndrome (RDS). Although essential to their survival, over time, volume and pressure overload from mechanical ventilation and excess oxygen are injurious to a newborn's lungs, leading to abnormal lung structure and function (64, 65). Premature lungs that develop BPD have an early inflammatory reaction and show increased levels of neutrophils, macrophages, and inflammatory cytokines such as IL-8, IL-6, and TGF-β1 (66–68). Reparative pneumocyte hyperplasia and proliferation of perivascular smooth muscle cells occurs, leading to pulmonary hypertension and fibroblast hyperplasia (69, 70). Furthermore, impaired alveolar development results in alveolar hypoplasia and a decrease in alveolar number and surface area (69–73). The effect of apoptosis on the distortion of the alveolar epithelium in BPD is not clear. Elevated apoptosis was found in AEC2 and bronchiolar cells in infants with BPD (74). Recently, Lukkarinen *et al.* (75) showed that preterm infants who had RDS and received mechanical ventilation had higher levels of apoptosis particularly in the epithelial cells. Increasing levels of epithelial apoptosis have been observed in neonatal mice exposed to high concentrations of oxygen (76) and in epithelial cells subjected to high levels of mechanical forces (35), two major contributing factors in the development of BPD. It seems that excessive

apoptosis, above physiologic levels, may worsen the disease, leading to ultrastructural abnormalities, and may be a potential culprit in reducing the number of alveoli characteristic of BPD. Excessive alveolar epithelial cell death could overwhelm the necessary elimination of proliferating mesenchymal and inflammatory cells from the alveolar air space or alveolar walls and further induce the proliferation of lung fibroblasts through the loss of normal inhibition, prolonging inflammation and interfering with re-epithelization, leading to fibrotic overgrowth of lung fibroblasts (77). TGF-β1 acts as a chemoattractant for fibroblasts (78) and stimulates immature fibroblast proliferation (79). Furthermore, TGF-β1 induces apoptosis in human lung epithelial lines (80) and enhances Fas-FasL–mediated apoptosis *via* caspase 3 activation and down-regulation of p21 expression, a cyclin-dependent kinase inhibitor, both *in vitro* (81) and *in vivo* (77). Increased levels of TGF-β1 are found in airway lavage samples of infants in the early stages of BPD (68). Moreover, high levels of TGF-β1 are associated with more severe cases of BPD (68). To date, the expression of FasL in infants with BPD has not been examined. However, given the predominant role of Fas-FasL in adult RDS (82, 83) and considering the occurrence of epithelial apoptosis in BPD, one may speculate on its involvement. Therefore, in BPD during tissue remodeling, dysregulation of the TGF-β1 pathway may act synergistically with Fas-mediated epithelial cell apoptosis during the pathogenesis of pulmonary fibrosis.

Paradoxic is that apoptosis is needed in the repair process of BPD during the fibrotic response. Apoptosis eliminates excess epithelial stem cells needed to reseal the denuded alveolar surface (84) and removes excess proliferating mesenchymal cells (85) and inflammatory cells, such as neutrophils, from the alveolar air space or alveolar walls (86). Failure to clear unwanted cells by apoptosis will prolong the inflammation as a result of the release of their toxic contents. Therefore, apoptosis may have both beneficial and detrimental effects during BPD. Incorrect timing, organization, and location of apoptosis during BPD may result in insufficient growth and disordered maturation of the lung.

CONCLUSION

The spotlight focusing on the importance of proliferation and differentiation during lung development has shifted to include apoptosis as an important key player. Normal lung development is associated with a progressive increase of apoptosis constantly counteracting proliferation. Intermittent FBM are essential for proper lung development and may act as a potential key regulator of apoptosis. Because prematurely born infants with immature lungs need continuous mechanical ventilation for their survival, the fine balance between growth and apoptosis may be compromised.

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