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Apoptosis of mesenchymal cells during the pseudoglandular stage of lung development affects branching morphogenesis

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Abstract

Apoptosis has been implicated as an important process in the development of several organ systems, including limbs, kidneys, and the heart. In developing murine lungs, we found that apoptosis was more predominant during the pseudoglandular stage of lung development than during the saccular stage with ninety-three percent of the apoptotic structures in the mesenchyme. Murine lung explants cultured in the presence of zinc chloride and aurointricarboxylic acid, two blockers of endonuclease function, showed decreased branching. These observations suggest that apoptosis predominates in mesenchymal cells during the pseudoglandular stage of lung development, and may be important for normal progression of lung branching morphogenesis.

INTRODUCTION

During embryogenesis, the mammalian lung undergoes a dramatic transformation from an amorphous mass of cells into a sophisticated organ with complex architecture and function [1]. The lung pattern is established very early and is dependent on differential regulation of the proliferation, differentiation, and migration of distinct groups of cells. Another process likely to contribute to pattern formation in lung is cell suicide or programmed cell death. One type of programmed cell death, *apoptosis*, is believed to play an important role in the regulation of animal cell populations during embryogenesis [2,3]. Morphologically, apoptosis is characterized by the detachment of cells from their surroundings, shrinking of the cytoplasm, chromatin condensation in the nucleus, and the sequestration of nuclear and cytoplasmic material into apoptotic bodies [4]. These bodies are phagocytized by neighboring cells where breakdown of the enclosed cellular material occurs by lysosomal enzymes [4,5]. Although not universal, the aforementioned morphological alterations are accompanied by the cleavage of genomic DNA into oligonucleotide fragments which results in the characteristic DNA ladder pattern observed by DNA electrophoresis [6,7]. Examples of developmental processes where apoptosis has been implicated include limb development [8] and metamorphosis of the tadpole [9]. In human embryogenesis, apoptosis has been described in the development of the heart [10], kidney [11], intrahepatic bile duct [12], and nervous system [5].

Apoptosis has been demonstrated in pulmonary cells of adult human lungs after injury [13], but less is known about how apoptosis is integrated in the complex program of lung development. In the prenatal period, murine lung development has 4 stages: embryonic, pseudoglandular, canalicular, and saccular. Each stage has specific morphologic characteristics that ultimately contribute to postnatal structure and function. Several studies have previously

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looked at apoptosis in the developing lung using different models, but the relationship with gestational age has been inconsistent. Using light and electron microscopy, Scavo et al. (1988) detected apoptosis in rat and human throughout the developmental stages, but no correlation was found with gestational age [14]. Kresch et al. reported that apoptosis was predominantly in the mesenchyme around distal airways in the fetal rat lung, and peak apoptosis occurs immediately after birth [15]. However, Stiles et al. found that while apoptosis occurs throughout embryonic development, there were two peaks of activity in the pseudoglandular and saccular stages. [16]. Levesque et al. (2000) showed a predominant mesenchymal population of apoptotic cells in lung explant cultures during branching morphogenesis when pattern formation occurs to establish the template for the development of the tracheobronchial tree [17].

In this report, we confirm the findings of Stiles et al. and Levesque et al. showing that even though apoptotic cells can be detected during the pseudoglandular and alveolar stages of lung development, they are most prominent during the pseudoglandular stage when branching morphogenesis occurs [16,17]. As Levesque et al. and Kresch et al. discovered, we also found that most cells undergoing apoptosis were found located within the mesenchyme [15,17]. We also extend their findings by demonstrating diminished branching in lung explants cultured in the presence of endonuclease inhibitors known to inhibit apoptosis. Overall, our work suggests that apoptosis during the pseudoglandular stage of lung development plays an important role in branching morphogenesis.

METHODS

Reagents

All reagents were purchased from Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

Isolation and processing of embryonic lungs

CD-1 pregnant females were sacrificed on D13 or D14 (pseudoglandular stage) and D17 or D18 (saccular stage) of gestation as previously described [18,19] and placed in Hank's Buffered Saline Solution (HBSS). Lungs to be used for immunohistochemistry were placed in 4% paraformaldehyde for 2h at room temperature, then 10% buffered formalin overnight at 4°C, dehydrated in ethanol, paraffin embedded, and sectioned (5 µm). Lungs used for DNA analysis were placed in THNE buffer (0.1 M Tris, pH 7.4, 0.1 mM EDTA, 2 M NaCl) and stored at -70°C until needed. Lungs used for the analysis of apoptosis by ELISA were placed in incubation media obtained from a cell death detection kit (Boehringer Mannheim, Germany) and stored at -70°C until needed.

Detection of apoptotic cells by enzyme-linked immunoassay (ELISA)

Cells from whole lung rudiments were obtained to reveal histone-associated DNA fragments by the Cell Death Detection ELISA Kit following the manufacturer's instructions (Boehringer Mannheim, Germany). This photometric enzyme immunoassay measures DNA fragmentation by utilizing the quantitative sandwich-enzyme-immunoassay principle. Mouse monoclonal antibodies directed against DNA and histones were used to determine the amount of mononucleosomes and oligonucleosomes in the cytoplasmic fraction of cell lysates. The cells were incubated in incubation buffer for 30 minutes at 4°C, then the lysates were centrifuged at 17,000 rpm (20,000 ×g) for 10 minutes. 400 µl of the supernatant containing the DNA and histones were carefully removed for analysis or stored at -80°C until needed.

Histology, TUNEL labeling, and morphometric analysis of lung tissue sections

Embryonic lungs were harvested and fixed in 4% paraformaldehyde for 2 hours at room temperature, transferred to 10% buffered formalin overnight at 4°C, dehydrated in decreasing concentrations of ethanol, xylene cleared, paraffin embedded, and sectioned (5 µm). The sections were stained with hematoxylin & eosin or immunostained and counterstained with hematoxylin. Direct immunoperoxidase staining was performed using the terminal transferase dUTP-digoxigenin nick end-labeling (TUNEL) method [20]. For this, paraffin embedded sections of embryonic lungs were heated to 60°C, placed in xylene, then in a series of ethanol solutions from 100% down to 70%. During the dehydration step, the sections were placed into a 3% hydrogen peroxide and methanol solution to quench endogenous peroxidases. Sections were washed in PBS, then placed into a protein kinase solution to better expose the endonuclease-nicked ends of DNA for coupling with the digoxigenin. Equilibration buffer was added directly to the section and allowed to incubate for 17–30 minutes. The buffer was shaken off and immediately the TdT enzyme was added and incubated at 37°C for one hour. Next the specimen was placed into a stop/wash solution to terminate the reaction. The sections were rinsed in PBS, incubated with an anti-digoxigenin-peroxidase complex for 30 min, rinsed, and developed with 0.5% 3,3'-diaminobenzidine-0.01% hydrogen peroxide solution in 0.1 M Tris pH 7.2 for 6 minutes.

Apoptotic structures were examined in multiple lung sections as follows. Using a light microscope and video imaging equipment, an image of each section was generated into a TV monitor. The lung area was calculated using the Image-1 Morphology Program (Universal Imaging Corp., West Chester, PA) by outlining the area of interest and calculating the 2 dimensional area. A total of 25 lungs were examined. From the group of D14 lungs, there were 14 lungs collected; 3–4 individual sections were obtained from each lung and stained for apoptotic cells and bodies. Five different 1 mm² areas chosen at random were assessed from each lung section with a total of 240 areas examined. The D18 lung group consisted of 11 lungs; 3–4 sections of each lung were assessed. Five different areas of each lung section were examined resulting in a total of 195 individual areas assessed for D18 data.

Immunohistochemistry was performed for phosphohistone H3 (Abcam, 1:200) using embryonic lung tissue fixed in 4% paraformaldehyde. 5 µm sections were rehydrated and underwent antigen retrieval and quenching of endogenous peroxidase activity. Sections were incubated with primary antibody at 4°C overnight. Incubation with the secondary antibody was performed using the Vectastain ABC kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions. DAB (Vector Labs, Burlingame, CA) was used for chromogenic visualization.

Lung cell proliferation assay

Total DNA content per lung was estimated using a previously described procedure [21,22]. Briefly, D14 and D18 lung rudiments were homogenized in 200 µl THNE buffer (0.1 M Tris, pH 7.4, 0.1 mM EDTA, 2 M NaCl), followed by freeze-thawing and sonication for 1 min. The samples (50 µl) were added to a cuvette containing 950 µl of THNE buffer plus 1000 µl of a 0.1 µg/ml Hoechst dye solution (bisBenzimide, cat#B-2883, Sigma, St. Louis, MO) and the fluorescence measured with a spectrofluorometer (Hoeffer Scientific, model TK0-100, San Francisco, CA). The total content of DNA per lung rudiment was calculated using a standard curve developed by measuring the fluorescence emitted by known concentrations of calf thymus DNA (Hoeffer, San Francisco, CO).

Detection of DNA endonuclease fragments in agarose gels

The detection of DNA endonuclease fragments in agarose gels was performed as previously described [23,24]. Embryonic mouse lungs (D14 and D18) were washed twice with 10 ml of

ice cold PBS and cells were lysed with 100 μ l Cell Lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.2 % Triton-X 100) for 30 min at 4°C. The lysates were centrifuged for 10 min and supernatants containing fragmented DNA were exposed to 100 μ g/ml RNase A for 1 hr at 37°C, and 100 μ g/ml Proteinase K for 2 hr at 37°C. An aliquot of prepared fragmented DNA was extracted in phenol/chloroform, re-extracted with chloroform, and the DNA was precipitated with 1 ml of ice cold 100% ethanol, centrifuged, dried, and resuspended in TE (pH 8.0) to later become radiolabeled for hybridization. Another aliquot of fragmented DNA (10 μ g) was added to DNA loading buffer (17 mM EDTA, 2% SDS, 0.1% Bromophenol Blue, 50 % glycerol), heated to 65°C for 17 min. and electrophoresed on 1% agarose gel in 1x TBE running buffer (0.045 M Tris-borate, 0.001 M EDTA) at 65 v for 4.5 hr. The DNA was denatured (1.5M NaCl, 0.5 M NaOH), neutralized (1.5M NaCl, 0.5 M Tris-HCl, pH 7.0) and transferred in 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) to nylon membrane, and then dried and UV crosslinked using a Fisher Scientific FB-UVXL-1000 crosslinker set at the optimal setting.

To create a mouse specific radiolabeled probe, 2 μ g of prepared fragmented DNA was labeled by the random prime method following the manufacturer's instructions (Stratagene, La Jolla, CA). The nylon membrane containing the fragmented denatured DNA was prehybridized (0.25 M Na₂HPO₄ pH 7.4, 1 mM EDTA, 20% SDS, 0.5% blocking Reagent) at 68°C for 2 hr, and then hybridized under the same conditions using 10 ng/ml radiolabeled probe (2 $\times$ 10⁶ cpm/ng). The membranes were washed twice with 30 ml of 0.2x SSC with 0.1% SDS for 17 min. at 42°C, and twice with 30 ml of 0.1x SSC with 0.1% SDS for 17 min. at 42°C, dried, and exposed to x-Ray film for 2 hr.

Lung explant culture and examination of lung branching morphogenesis

The age of the embryos was estimated from day 0 when the vaginal plug was found. Lung rudiments were dissected at 11 days of gestation and maintained *in vitro* in the upper level of a culture chamber (Costar, Cambridge, MA) at the air-liquid interface as previously described [18,19]. The lungs were maintained at 37°C for 4 days in medium alone or in the presence of the endonuclease inhibitors zinc chloride (Zn, 50 μ M) and aurintricarboxylic acid (ATA, 10 μ M). The experimental reagents were suspended in Dulbecco's Modified Eagle's Medium and identical volumes of each experimental reagent were added to the experimental samples once every day. Regular media was added to the controls.

The lung rudiments were examined daily and the number of branch points counted. Branch points were defined as areas of airway bifurcation or cleft formation including the primordial carina [18,19]. Because cleft formation and the development of new branches depends on the original number of branches at day 11, we calculated the fold increase in branch points (i.e., the number of branch points at culture day 4, 17 days of gestation, divided by the number of branch points at culture day 1, 11 days of gestation). Differences in fold increase in branch points between control and experimental samples were analyzed initially by ANOVA, and treatment groups showing significant differences were determined by Student's -t-test. Measurement of mesenchymal tissue was performed using image analysis program Image J (NIH) on photos of the lung explants taken at the end of the culture period. The area within each cleft and between adjoining buds was outlined to quantitate the pixel area of mesenchymal tissue.

Immunofluorescence in lung explants

After 5 days in culture, lung explants were fixed in 4% paraformaldehyde overnight at 4°C. The explants were initially washed in DMSO, and then were washed with PBS using several washes. Blocking with donkey serum (Jackson Immunolab) was done for 3 hours. The explants were then incubated overnight at 4°C with primary antibody against FGF-10 (Abcam, 1:400).

Several washes were then performed using PBS. The explants were then incubated with a rhodamine tagged secondary antibody (Jackson Immunolab, 1:250) overnight at 4°C. Staining was visualized using a Zeiss confocal microscope.

Statistical Analysis

Experimental data from multiple experiments (at least three) underwent statistical analysis using $P < 0.05$ as the cutoff for statistical significance. Data is presented as mean $\pm$ SD. The Student's t-test and ANOVA were used as appropriate.

RESULTS

Detection of apoptosis and cell division

Apoptosis in embryonic murine lung was detected using several methods. First, we examined for apoptosis by electrophoresis of total DNA obtained from whole D14 and D18 lungs. Figure 1A demonstrates the presence of more fragmented DNA in the lung extracts of D14 lungs when compared to the D18 lungs. This figure depicts studies performed on two samples of D14 lungs and two samples of D18 lungs that were extracted and submitted to electrophoresis on a 1% agarose gel using equal loading of 10 μ g of DNA/lane. This somewhat crude method suggested that apoptosis occurs in a dynamic fashion depending on the particular stage of development.

The finding that apoptosis was more predominant in the pseudoglandular stage was corroborated using a modified enzyme-linked immunoassay that detects fragmented DNA in whole lungs. The data were normalized for total DNA per lung, which was determined by DNA fluorometry (Figure 1B). The figure shows that, consistent with the observations made by DNA electrophoresis, D14 lungs ($n=6$) contain approximately 2.2 times more apoptotic cells per explant than D18 lungs ($n=6$).

To determine the specific cell types undergoing apoptosis, 5 μ m tissue sections of D14 and D18 murine lungs were examined using terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) to detect free 3' OH groups of fragmented DNA (ApopTagTM Plus, Oncor, Inc., MD). Similar to the observations described above, both D14 and D18 lungs contained cells undergoing apoptosis. Morphometric analysis of 87 tissue sections including 435 separate 1 mm² areas revealed that lungs obtained at D14 contained more apoptotic cells and bodies than D18 lungs (mean $\pm$ SE, 11.44 ± 3 vs. 6.02 ± 0.92 structures/mm² lung area) (Figure 1C).

Detailed examination of the tissue revealed that 93% of the apoptotic bodies and cells were detected within the mesenchyme of the embryonic lungs. Most apoptotic cells and bodies in D14 pseudoglandular lungs were detected within the mesenchyme predominantly around the primitive airways (Figure 2A and B). Although less frequent, apoptosis did occur in epithelial cells (Figure 2C). Figure 2D shows liver tissue stained for apoptosis as a positive control. In D18 saccular stage lungs, apoptotic cells remain mainly in mesenchymal tissue including vascular structures (Figure 2E and F), although the developing alveolar septa have a few apoptotic cells as well (Figure 2G). Control placental tissue and liver tissue is presented in Figures 2D and 2H as a positive controls. At D17, serial sections stained for TUNEL (Figure 2I) and fibronectin (Figure 2H) demonstrate that the TUNEL positive cells are mostly in the mesenchyme since fibronectin staining demarcates the epithelial basement membrane to separate the epithelial and mesenchymal compartments.

Cell division was evaluated using staining for phosphohistone H3. There appears to be less cell division per field at D17 when compared to D13 at the same magnification (40X) (Figures 3A and B). At D13, cell division appears to occur equally in the epithelium and mesenchyme, but at D17, cell division appears to slightly favor the mesenchyme (Figure 3C).

Effect of endonuclease inhibitors on lung branching morphogenesis

Since apoptosis appears more predominant during the time of lung branching morphogenesis, we treated cultured D11 lung explants with inhibitors of internucleosomal DNA fragmentation shown to inhibit apoptosis in other systems (see Discussion). These agents, Zn and ATA, were added daily to the cultures at 50 μ M and 5 μ M, respectively, based on doses reported to be optimal in other systems. Both inhibitors caused a significant reduction in the number of total branch points developed (Figure 4A) and a reduction in the fold increase in branching (Figure 4B). The inhibitory effect of the reagents was not greatly enhanced when both were added simultaneously.

The endonuclease inhibitor treated lungs had significantly more mesenchymal tissue when area between clefts was measured using image analysis (Figure 5). The examination of apoptosis by ELISA in cultured lung explants treated with the endonuclease inhibitors for 48 hours revealed that Zn-treated lung explants had only ~63% of the apoptosis detected in untreated lung explants. ATA-treated lungs, on the other hand, showed little decrease in apoptosis (92% of control), whereas lungs treated with Zn and ATA simultaneously showed the lowest amount of apoptosis (33% of control). This loss of apoptosis may explain the apparent increase in mesenchymal tissue. In addition, ATA-treated lung explants have less staining for FGF-10, an essential morphogen for branching morphogenesis, at the distal buds when compared to untreated control explants (Figure 6).

DISCUSSION

Apoptosis in pseudoglandular stage lungs

Apoptosis has been implicated in many biological processes ranging from angiogenesis and tumor growth [25] to developmental processes of the limb [8], heart [10], kidney [11], intrahepatic bile duct [12], and nervous system [5]. With regards to the embryonic lung, apoptosis has been detected in fetal rat (D18–22) and human (17–24 weeks of gestation) lungs obtained during the prenatal period, but there are conflicting data linking apoptosis to gestational age. Scavo et al. reported that apoptosis occurs throughout gestation, but did not find any association with the stage of lung development in rat and human lungs [14]. In a similar rat lung model, Stiles et al. agreed that apoptosis occurs throughout gestation, but also found that apoptosis appeared to be more prominent in the pseudoglandular stage as well as during late lung development, and was associated with the expression of pro-apoptotic factors [16]. Other literature available has also implicated apoptosis in postnatal lung development. In the postnatal period when functional alveoli are formed, increased apoptosis in type II epithelial cells and fibroblasts has been detected [26,27]. Fibroblasts isolated from postnatal rat lungs undergoing apoptosis were associated with alterations in the ratios of certain members of the bcl-2 gene family known to regulate apoptosis. In particular, increased apoptosis was associated with a decrease in bcl-2 mRNA (known to suppress apoptosis) that paralleled an increase in BAX homodimer mRNA (known to promote apoptosis) [26]. Another mediator of apoptosis, Fas, and its ligand, FasL, have been detected in developing mouse embryos from D18.5 onwards in several organs including lung [28]. It has been speculated that apoptosis is an important mechanism in the process of alveolarization perhaps by controlling the removal of excess mesenchymal cells during postnatal lung development [29].

Our work focused on the pseudoglandular stage of development when branching morphogenesis occurs. Although there are limitations in the methods we used to detect apoptosis, the results consistently suggested that apoptosis was more common in the pseudoglandular stage than late gestation. Our studies revealed more apoptosis (~2x) in pseudoglandular stage lungs when compared to alveolar stage lungs. This finding was consistent independent of the methodology used to test apoptosis including the detection of

DNA fragmentation by gel chromatography and ELISA, and histological analysis of tissues stained using the TUNEL method. Taking into account the number of cells present in pseudoglandular stage murine lungs (as determined by DNA fluorometry), we estimate that the number of cells undergoing apoptosis in these lungs at any given time is 1–2%. Our findings that apoptosis peaks during the pseudoglandular stage of development correlates with the findings that Stiles et al. reported in the developing rat lung. We did not find a peak in apoptosis in the late lung development during sacculation and most studies have not found apoptosis to be particularly significant during the sacculation stage. During this stage, it appears that proper vascular formation is one of the driving forces to begin creating adequate surface area for gas exchange. Zhao et al. demonstrated that interfering with VEGF function results in impaired sacculation with increased apoptosis and decreased proliferation which supports previous findings that lack of apoptosis during sacculation is probably desired [30]. Apoptosis may occur more in the pseudoglandular stage because of the structural change from a rounded bud to cleft formation. In contrast, apoptosis may be less important during sacculation because the airspaces themselves are growing and this enlargement may have a mechanical effect on compressing and thus thinning the mesenchyme

Since other morphologic phenomenon such as limb and kidney formation rely on apoptosis, it is reasonable to expect that apoptosis plays a role in formation of the tracheobronchial tree by epithelial branching morphogenesis in the lung [1,31]. Our finding that apoptosis occurs in the mesenchyme during the pseudoglandular stage when pattern formation occurs supports this hypothesis and is concordant with the previous findings of Kresch and colleagues. Kresch et al. found that apoptosis in the developing rat lung occurred predominantly in the mesenchyme around distal airways until late gestation when epithelial apoptosis began to emerge [15]. Pattern formation is thought to be driven by the balancing forces of *inductive* signals that maintain epithelial cells proliferating at the tips of the growing bud, and *negative* signals that diminish epithelial cell proliferation in other areas thereby creating clefts or branching points (reviewed in 1). The epithelium and mesenchyme both contain these signals and the process is coordinated by a dynamic temporospatial program. Since 93% of the apoptotic cells were found in the mesenchyme, apoptosis itself may be involved in the dichotomy that develops between the epithelium and mesenchyme during branching morphogenesis. With the formation of the tracheobronchial tree, the supporting structure is also created for vascular development and ultimately the gas exchanging alveolar-capillary units. The mesenchyme “thins” to accommodate the developing conductive airways and alveolar spaces while the epithelium continues to proliferate, and apoptosis may play a role during this process. This is reminiscent of work that implicates apoptosis in the removal of excess mesenchymal cells in resolving pulmonary lesions [13].

In support of the idea that mesenchymal cell apoptosis might be important for branching morphogenesis, we found that inhibitors of endonuclease activity could diminish branching in cultured lung explants. Two agents were used for these experiments. The first, Zn, caused a decrease in branching that was related to a concomitant decrease in apoptosis as measured by ELISA. We cannot prove that Zn works solely through the prevention of apoptosis. However, there is strong evidence in the literature that supports its role in this process. For example, it has been demonstrated that normal development requires the presence of Zn in most, if not all, animal species [32]. The administration of Zn-deficient diets to animals results in increased apoptosis in many organs mainly because there are no stores of Zn in the body, and Zn homeostasis is regulated largely by intestinal absorption and excretion [33]. This trace element serves as a cofactor, coenzyme, or protein structural component for a wide range of biological functions including DNA synthesis and sequence recognition, transcriptional regulation, cytoskeletal and membrane integrity, and cellular energy metabolism, all mechanisms that could trigger and/or modulate programmed cell death [33,34]. How Zn regulates apoptosis is not entirely clear. Zn deficiency can affect cells indirectly as was demonstrated in rodents

where Zn deprivation induced excessive levels of circulating glucocorticoids [35]. However, studies *in vitro* show that Zn deficiency has direct effects on many systems. The Ca/Mg-dependent endonuclease that cuts linker regions between nucleosomes leading to DNA fragmentation is sensitive to Zn concentrations [7]. Caspases, proteases that cleave substrates capable of initiating the pro-apoptotic cascade, may also be affected by Zn [36]. In this regard, two of the known caspases (caspase 3 and 6) appear to be targets for Zn [37,38]. Although we did not look at caspase activity, it likely plays a role in our findings. Bustani et al. demonstrated that in a human pseudoglandular stage lung explant model of hyperoxia, increased activated caspase 3 levels were associated with increased apoptosis in the interstitium with thinning and a more saccular stage phenotype [39]. Fanzo et al. showed that zinc supplementation reduces caspase 3 levels in human bronchial epithelial cells when compared to cells with a normal zinc concentration [40]. Thus, the zinc concentration used in our studies may have been high enough to reduce caspase levels or activity. Zn has also been implicated in the regulation of Bcl-2-like membrane proteins also known to modulate apoptosis [41,42].

Our results with ATA are more difficult to interpret. ATA is a triphenylmethane dye that is thought to act by interfering with the formation of protein-nucleic acid complexes [43]. ATA inhibits DNase, RNase A, S1 nuclease, exonuclease III, and restriction endonucleases SaI I, Bam HI, Pst I, and Sma I *in vitro* [44]. Like Zn deprivation, ATA has been used extensively to inhibit apoptosis [45–47]. Although ATA diminished the branching of cultured lung explants, we could not demonstrate significant inhibition of apoptosis by ELISA, but it did enhance apoptosis when provided with Zn. Other tests are necessary before we arrive to definitive conclusions. However, this discrepancy may be explained by observations showing that this agent is known to affect only some types of endonucleases, whereas Zn has broader activity. It is also possible that ATA affects apoptosis in few specific cells that are crucial for the process of branching. Our data showing a decrease in FGF-10 staining in the ATA treated lung explants suggests that ATA may also be affecting the inherent branching morphogenesis program of morphogen expression which includes proper expression of FGF-10 at the bud tips. However, further work needs to be done to show that other morphogen expression is also decreased and that the decreased FGF-10 staining is not simply because less mesenchyme is present.

Our findings contrast with previously published work by Kling et al. which showed that inhibiting MEK resulted in increased apoptosis in the mesenchyme of rat lung explants but decreased branching. Although Kling et al reported diminished branching with increased apoptosis, the impact on branching with MEK inhibitors is likely more complex. The MAPK pathway has been reported as an important downstream intracellular event triggered by growth factor receptor activation by ligands, including fibroblast growth factor and epidermal growth factor. Both of these growth factors are important in organ morphogenesis and inhibition of their receptor activation inhibits morphogenesis. Use of MEK inhibitors would also affect the downstream events of growth factor receptor activation and this may overshadow any positive effects that increased mesenchymal apoptosis may have [48,49].

In summary, our studies suggest that apoptosis predominates in lung mesenchymal cells during the pseudoglandular stage of lung development and inhibition of apoptosis adversely affects branching morphogenesis.

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Figure 1. Apoptosis predominates in mesenchymal cells during the pseudoglandular stage of lung development

A. Detection of apoptosis in developing lungs using autoradiography. We examined apoptosis by electrophoresis of total DNA obtained from D14 and D18 murine lungs. The entire lungs were extracted followed by DNA electrophoresis, probing and autoradiography. The figure depicts a gel with four lanes. The first two lanes contain DNA extracted from D14 lungs, whereas the other two lanes contain DNA extracted from D18 lungs. The dark smear present in the first two lanes indicates more fragmented DNA in the D14 lung extracts. **B. Detection of apoptosis in developing murine lungs by ELISA.** Apoptosis was examined using a modified enzyme-linked immunoassay to detect fragmented DNA in the extracts of whole lung preparations. These data were normalized for total DNA per lung that was determined by DNA fluorometry. Note that D14 lungs contained more apoptotic structures (~2x) than D18 lungs. **C. Morphometric analysis of lung tissue for apoptosis.** A total of 25

lungs were examined (D14: 14 lungs; D18: 11 lungs). Lung tissue blocks were sectioned (5 μm) and three slides of each lung were examined. Five different 1 mm² areas were analyzed per lung. Using light microscopy, a video image of each section was generated on a TV monitor and the lung area was estimated using a computer program for morphometry. Analysis of the data revealed that D14 lungs contained more apoptotic cells and bodies than D18 lungs.

Figure 2. Detection of apoptotic cells in D14 and D18 lungs by TUNEL
A and E. Predominance of apoptotic structures in mesenchymal tissues (10x magnification). Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) was used to specifically detect free 3' OH groups of fragmented DNA (ApopTag™ Plus, Oncor, Inc., MD) in embryonic lungs. D14 and D18 lungs were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned (5 μm). Few apoptotic cells and bodies were detected per field by TUNEL. At D14, most of these cells were detected within the mesenchyme (m) (arrows), but few were also detected in the epithelium (e) (Figure 2A). At D1, most cells were present within mesenchymal structures including vessels (v) (arrows) (Figure 2E). **B and F. Proximity of apoptotic cells to airway**

structures (20x magnification). Apoptotic cells and bodies present within the mesenchyme (m) were often found in clusters near airway structures (arrow) at D14 (Figure 2B). Apoptotic structures could be detected in septa between developing alveoli (a) at D18 (Figure 2F). **C and G. Apoptosis in the epithelium (20x magnification).** Although some apoptosis was detected in the epithelium (e), most apoptotic structures were detected within the mesenchyme (arrows) at both D14 (Figure 2C) and D18 (Figure 2G). **D and H. Apoptosis in liver (10x).** Liver sections from D14 (Figure 2D) and placenta from D18 (Figure 2H) embryos were examined for apoptosis as control. Notice the presence of apoptotic cells in embryonic liver tissue. **I and J. Apoptosis in the mesenchyme (40x magnification).** At D17, apoptosis occurs in the mesenchyme with serial sections of fibronectin expression. The epithelium is demarcated by fibronectin expression in the basement membrane.

Figure 3. Proliferation during lung development. A and B. Proliferation at D13 and D17 Phosphohistone H3 staining demonstrates that cell proliferation decreases over time during lung development, with the highest number of positive staining cells at D13 (A) when compared to D17 (B). **C. Distribution of proliferation.** During early lung development at D13, proliferation occurs almost equally in the epithelium and mesenchyme, but then becomes more predominant in the mesenchyme at D17.

Figure 4. Effect of inhibitors of apoptosis on lung branching morphogenesis

To examine the potential role of apoptosis in lung branching morphogenesis, we cultured D11 lung explants for 4 days in the presence and absence of the endonuclease inhibitors Zinc (Zn) and Aurintricarboxylic Acid (ATA). **A. Reduction in branch points.** Both inhibitors caused a significant reduction in the number of total branch points developed during culture. * $p < 0.02$, † $p < 0.005$. **B. Reduction in fold increase in branching.** A reduction in fold increase in branching when compared to control lungs was also noted. The inhibitory effect of the reagents was not greatly enhanced when both were added simultaneously suggesting similar mechanisms of action.

Figure 5. Effects of the inhibitors on lung morphology

A and B. Decreased branching with inhibitors of apoptosis. D11 lung explants were cultured for 4 days in the absence (A) or presence (B) of the endonuclease inhibitor Zinc. Afterwards, the lungs were harvested to examine morphology. Examination of the lung explants by light microscopy revealed less branches in the Zn-treated rudiments when compared to control. **C. Increased mesenchyme with inhibitors of apoptosis.** The Zn-treated lungs showed a significant increase in mesenchymal tissue (arrows in A, B) in branching clefts. * $p < 0.01$

Figure 6. Decrease in branching correlates with decreased FGF-10 expression

In D11 lung explants cultured for 4 days in the absence (A) or presence (B) of aurintricarboxylic acid (ATA), expression of FGF-10 was determined using immunofluorescent staining. FGF-10 staining was decreased at the bud tips (b) and in the branching clefts (c) in the presence of ATA, correlating with the decreased branching previously observed.