Injury, inflammation, and remodeling in fetal sheep lung after intra-amniotic endotoxin

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Kramer, Boris W., Susanne Kramer, Machiko Ikegami, and Alan H. Jobe. Injury, inflammation, and remodeling in fetal sheep lung after intra-amniotic endotoxin. Am J Physiol Lung Cell Mol Physiol 283: L452–L459, 2002. First published March 22, 2002; 10.1152/ajplung.00407.2001.—Chorioamnionitis is frequent in preterm labor and increases the risk of bronchopulmonary dysplasia. We hypothesized that intra-amniotic endotoxin injures the lung in utero, causing a sequence of inflammation and tissue injury similar to that which occurs in the injured adult lung. Preterm lambs at 125 days gestational age were evaluated for indicators of inflammation, injury, and repair 5 h, 24 h, 72 h, and 7 days after 4 mg of intra-amniotic endotoxin injection. At 5 h, the epithelial cells in large airways expressed heat shock protein 70, and alveolar interleukin-8 was increased. Surfactant protein B (SP-B) decreased in alveolar type II cells at 5 h, and SP-B in lung tissue and alveolar lavage fluid increased by 72 h. By 24 h, neutrophils were recruited into the large airways, and cell death was the highest. Alveolar type II cells decreased by 25% at 24 h, and proliferation was highest at 72 h, consistent with tissue remodeling. Intra-amniotic endotoxin caused surfactant secretion, inflammation, cell death, and remodeling as indicators of lung injury. The recovery phase was accompanied by maturational changes in the fetal lung.

surfactant; lung maturation; alveolar type II cells; respiratory distress syndrome; chorioamnionitis

CHORIOAMNIONITIS is frequently associated with preterm birth, with indicators of systemic and pulmonary inflammation in the newborn at birth and with increased risks of bronchopulmonary dysplasia and brain injury (cerebral palsy) (9, 17, 20). In contrast, histological chorioamnionitis is also associated with a decreased risk of respiratory distress syndrome and may predict increased survival of preterm infants (6, 25). With the use of a lamb model to study fetal lung responses to chorioamnionitis induced by intra-amniotic endotoxin, we found that the lung responded by recruiting proinflammatory cells that produced hydrogen peroxide (15, 16). Proinflammatory cytokine mRNAs were expressed in the lung 5 and 24 h after intra-amniotic endotoxin. Subsequently, there was a striking maturational effect characterized by improved lung mechanics and gas exchange and large increases in surfactant pool sizes within 7 days of the intra-amniotic endotoxin (1, 13, 14). The maturational effects on the fetal lung were accompanied by an arrest in alveolarization (26). Inflammatory cells that were not producing hydrogen peroxide or cytokine mRNA persisted in the lungs for several weeks, indicating ineffective clearing of the inflammatory process (15, 16). There was a minimal systemic response to the intra-amniotic endotoxin with no increase in fetal cortisol levels or in cytokine expression in liver, gut, or placental tissue (13, 15). Low doses of intra-amniotic endotoxin that did not induce a maturational response also did not increase indicators of inflammation in the fetal lung (16). We hypothesize that intra-amniotic endotoxin initially induces a lung injury response in fetal sheep lung. We evaluated injury responses to endotoxin characteristic of lung injury in the adult. We assessed induction of heat shock protein 70 (HSP70) as an indicator of oxidant injury (19, 28), interleukin (IL)-8 as a mediator of neutrophil recruitment (17), and the loss of surfactant protein B (SP-B) from alveolar type II cells as an indicator of injury to alveolar type II cells (29). Apoptosis, proliferation, and alveolar type II cell numbers were evaluated as indicators of remodeling of the fetal lung.

MATERIALS AND METHODS

Animals, study design, and endotoxin. The animal studies were performed in Western Australia, as approved by the Animal Care and Use Committees from the Cincinnati Children’s Hospital and the Western Australian Department of Agriculture. Date-bred Merino ewes were randomized to receive 4 mg of endotoxin at 5 h, 24 h, 72 h, or 7 days before delivery at 125 days gestational age (Fig. 1). The 4-mg dose was the lowest dose that gave consistent maturational responses (16). Endotoxin (Escherichia coli O55:B5; Sigma Chemical, St. Louis, MO) was solubilized in saline and filtered through a 0.45-μm filter. Intra-amniotic saline or endotoxin injections were given with 20-gauge spinal needles with ultrasound guidance (13, 14). The inflammatory responses in the chorioamnion, amniotic fluid, and lung tissue were recently reported using samples from the same animals (16).

Delivery and postnatal assessment. Preterm lambs were delivered at 125 days gestational age by cesarean section (7, 12). The thorax was opened, and the top right lobe of the lung was inflation fixed with 10% formalin at 30 cmH2O pressure.

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The left lung was lavaged as previously described (24). Five alveolar lavages of the left lung with 0.9% NaCl at 4°C were pooled, and aliquots were saved for determination of saturated phosphatidylcholine (Sat PC), IL-8, and SP-B (7, 24). A piece of the lavaged left lung was frozen.

**Immunohistochemistry and histological analyses.** Lung tissue was formalin fixed, embedded in paraffin, and cut in 5-μm sections. Endogenous peroxidase activity was removed by incubation with hydrogen peroxide. Nonspecific binding sites were blocked with serum. Sections were incubated with a rabbit polyclonal antibody for HSP70 (Santa Cruz Biotechnology, Santa Cruz, CA). After being washed with PBS to remove unbound antibody, the slides were incubated with the secondary biotinylated antibody against rabbit IgG (Vector Laboratories, Burlingame, CA). After adding avidin and horseradish peroxidase (Vector Laboratories), staining of HSP70-positive cells was developed with diaminobenzidine and cobalt with a nuclear fast red counterstain (21).

Alveolar type II cells were stained after antigen retrieval with a mouse monoclonal antibody specific for thyroid transcription factor-1 (TTF-1) as previously described (11, 21, 22). The antibody was kindly provided by Dr. J. A. Whitsett. The SP-B in the alveolar type II cells was evaluated by immunohistochemistry using a rabbit anti-SP-B that reacts with both completely processed and unprocessed forms of SP-B (1). Sections were graded for the number of SP-B-positive cells using a scale from 0 to 4. For each triplicate section from each animal, 12 areas were randomly selected and evaluated.

**Fig. 1.** Study design: intra-amniotic injections of 4 mg of endotoxin were given 5 h, 24 h, 72 h, and 7 days before delivery at 125 days (d) gestational age (GA). Intra-amniotic injections are marked with arrows. The numbers of animals/group are given in parentheses.

**Fig. 2.** Heat shock protein 70 (HSP70) and inflammatory cells. A: HSP70 was expressed at 5 h in the epithelial cells of the fetal airways but not in the parenchyma. B: at 24 h, no HSP70 was detectable. C: hematoxylin and eosin staining of fetal lung after 5 h of endotoxin exposure. D: inflammatory cells in epithelium of large airways at 24 h after intra-amniotic endotoxin are indicated by arrow. The cells were predominantly neutrophils. Magnification ×320.

**Fig. 3.** Interleukin (IL)-8. IL-8 concentration was measured in alveolar lavage fluid by ELISA and standardized per kilogram of body wt. IL-8 increased at 5 h and at all later time points vs. control. At 7 days, IL-8 increased further (*P < 0.05 vs. control, **P < 0.05 vs. 72 h).
for inflammatory changes, such as neutrophil influx, in the airways.

IL-8. The potent neutrophil chemoattractant IL-8 was quantified by enzyme-linked immunosorbent assay using antibodies from Chemicon (Temecula, CA) (16). Recombinant protein as a standard was kindly provided by CSIRO (Parkville, Victoria, Australia). The level of detection was 0.5 ng/ml.

Quantification of SP-B and Sat PC. SP-B was measured in alveolar lavage fluid and in lung tissue after alveolar lavage. Lung tissue was homogenized in 10 mM Tris, pH 7.3, and 2 M sucrose (vol/vol 7:1) to which protease inhibitors were added (Protease Cocktail; Roche Molecular Diagnostics, Indianapolis, IN). Homogenates were centrifuged for 10 min at 500 g at 4°C, and the supernatant was used for SP-B measurement by Western blots as previously described (1). SDS-PAGE for SP-B was carried out using 10–20% gradient tricine gels (Novex, San Diego, CA). Samples containing 3 nmol of Sat PC were electrophoresed along with an ovine SP-B standard under nonreducing conditions (1). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Schleicher & Schuell, Keene, NH) for immunoblot analysis using a rabbit anti-SP-B that reacts with both completely processed and unprocessed forms of SP-B (1). The PVDF membrane was first blocked with 5% low-fat milk (wt/vol) in Tris-buffered saline, pH 7.4, containing 0.1% (vol/vol) Tween 20. The antibody was diluted and incubated overnight. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Calbiochem, La Jolla, CA) was used as secondary antibody, and enhanced chemiluminescence substrates (Amersham, Arlington Heights, IL) were used to develop the immunoblots. Separate estimates of the amount of fully processed and partially processed SP-B were made by calculation of the relative band densities using Alpha-Imager 2000 documentation and Analysis software (Alpha Innotech, San Leandro, CA). Mature SP-B was calculated per kilogram

Fig. 4. Surfactant protein B (SP-B) in alveolar type II cells and in the lung. A: sections were stained with an antibody that reacts with processed and unprocessed SP-B. Representative sections for each time point are shown. Magnification ×320. B: immunostaining for SP-B was graded from 0 to 4. Intra-amniotic endotoxin reduced the SP-B content of type II cells within 5 h. At 72 h and at 7 days after intra-amniotic injection of endotoxin, the score for SP-B staining was higher than in controls. C: quantification of total (fully processed and partially processed) SP-B in total lung by Western blot expressed relative to a normalized value of 1.0 for the control group. At 72 h and at 7 days, the amount of total SP-B increased (*P < 0.05 vs. control).
of body wt in the alveolar lavage fluid and in the lung homogenate. Percent secretion of mature SP-B was calculated by dividing the amount in the alveolar lavage fluid by the sum of mature SP-B in alveolar lavage fluid and lung homogenate.

Sat PC was measured in alveolar lavage and lung tissue after lavage. Sat PC was isolated from chloroform-methanol (2:1) extracts by neutral alumina column chromatography after exposure of lipid extracts to osmium tetroxide (18). Sat PC was quantified by phosphorus assay (3).

**Apoptosis and alveolar type II cell quantification.** Apoptotic cells were detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling using biotin-dUTP (TACS 2 TdT-DAB kit; Trevigen, Gaithersburg, MD). Alveolar type II cells were identified by staining with a monoclonal antibody against TTF-1 (see Immunohistochemistry and histological analyses). The percentages of apoptotic cells and alveolar type II cells were quantified relative to all cells of the lung parenchyma. Intra-amniotic endotoxin injection induced both lung maturation and an arrest of alveolar septation, which reduced the volume of the parenchyma and increased the lung gas volume within 7 days (14, 26). The number of alveolar type II cells was not quantified after intra-amniotic endotoxin exposure. The quantification of alveolar type II cells relative to lung volume will be influenced by changes in the parenchyma to air space volume. Therefore, we quantified the apoptotic cells and alveolar type II cells relative to all cells by counting nuclei. Five sections for each animal were quantified.

**Proliferation.** Proliferating cell nuclear antigen (PCNA) is a nonhistone protein found in nuclei in the S, G2, and M phases of cell mitosis (8). Staining for PCNA was performed with a biotinylated monoclonal antibody (Zymed Laboratories, San Francisco, CA) and was developed with diaminobenzidine and cobalt with a nuclear fast red counterstain. The percentage of proliferating cells was quantified relative to all cells of the lung parenchyma.

**Data analysis.** Results are given as means ± SE. Comparisons between endotoxin-treated and untreated lambs were by analyses of variance with Student-Newman-Keuls tests used for post hoc analyses. Significance was accepted at $P < 0.05$.

**RESULTS**

**HSP70, inflammatory cells, and IL-8 as indicators of lung injury.** HSP70, an indicator of oxidant-stress mediated lung injury, was not expressed in control animals. In endotoxin-treated animals, HSP70 stained intensely in the epithelium of the conducting airways but not in the alveolar or vascular structures at 5 h.

![Fig. 5. Alveolar type II cells.](image)

Fig. 5. Alveolar type II cells. **A:** alveolar type II cells were stained for thyroid transcription factor-1. Representative sections are shown for controls and lungs 24 h and 7 days after endotoxin exposure. Magnification ×320. **B:** number of alveolar type II cells was determined relative to all cells in the lung parenchyma by counting nuclei. The percentage of alveolar type II cells was reduced at 24 h. At other time points, the numbers were not different (*$P < 0.05$ vs. control).
By 24 h, there was no expression of HSP70 detectable in epithelial cells lining the large airways or in the distal lung parenchyma (Fig. 2B), and no HSP70 expression was detectable at 72 h and at 7 days. At 5 h, there were no inflammatory changes in the large airways or in the distal lung parenchyma (Fig. 2C). By 24 h, the large airways were infiltrated with inflammatory cells, most of which were neutrophils based on their appearance at high magnification (Fig. 2D). Neutrophils were also present in the alveoli. The inflammatory cells persisted at 72 h and at 7 days. There were no inflammatory cells in the lungs of control animals.

**SP-B and alveolar type II cell numbers.** Immunohistochemistry demonstrated that SP-B was lost from alveolar type II cells 5 h after intra-amniotic endotoxin exposure (Fig. 4, A and B). This depletion was no longer present at 24 h, and more SP-B containing type II cells was identified at 72 h and at 7 days. The total amount of SP-B in the alveolar wash and lung tissue decreased qualitatively at 5 h and then increased 4.6-fold by 7 days (Fig. 4C). The ratio of secreted mature SP-B in the airspace to total SP-B increased from $10.4 \pm 2.0\%$ in controls to $26.1 \pm 5.2\%$ at 5 h ($P < 0.05$). The percent of SP-B in the air spaces increased to $62.7 \pm 8.5\%$ at 7 days ($P < 0.05$ vs. control and 5 h). The loss of SP-B from the tissue is consistent with the decreased immunostaining for SP-B.

The number of alveolar type II cells identified by TTF-1 staining as a proportion of total cells was not changed 5 h after intra-amniotic endotoxin exposure (Fig. 5). At 24 h, the percent of alveolar type II cells was reduced by ~25%, and at 72 h and at 7 days, the number of type II cells was not different from control animals.

**Tissue repair by apoptosis and proliferation.** The percent of apoptotic cells and the cell populations undergoing apoptosis changed over time. In controls and at 5 h, few cells were apoptotic (Fig. 6A). At 24 h, the highest percent of apoptotic cells was identified (Fig. 6B). Inflammatory cells in both the air spaces and epithelial cells were apoptotic. By 72 h and 7 days, fewer parenchymal cells were apoptotic, and the apoptotic cells were primarily inflammatory cells.

To assess proliferation, lung sections were stained for PCNA, a nonhistone protein elevated in the S, G2, and M phases of mitosis, and the cells were quantified (Fig. 7). There was no increase in proliferating cells as indicated by PCNA staining 5 or 24 h after intra-amniotic endotoxin exposure. At 72 h and at 7 days, the number of proliferating epithelial cells was increased.
DISCUSSION

We found that intra-amniotic endotoxin induces injury, inflammation, apoptosis, and remodeling of the fetal lung. A lung injury response was indicated by the expression of HSP70 in the epithelial cells of the large airways and depletion of SP-B from alveolar type II cells at 5 h. The percent of alveolar type II cells decreased at 24 h but was not different from controls at 7 days. Apoptotic cell death was maximal at 24 h, and cell proliferation was maximal at 72 h. We previously reported that intra-amniotic endotoxin caused recruitment of hydrogen peroxide-producing neutrophils to the fetal lungs and proinflammatory cytokine expression by cells recovered by alveolar lavage (15, 16). The identification of HSP70 in the large airway lining cells at 5 h indicates an oxidant injury response of the tissue (19, 28). Although hydrogen peroxide production was maximal at 24 h (16), the HSP70 response occurred at 5 h and was localized to the airways. Cells in the amniotic fluid contained increased IL-8 mRNA within 5 h of endotoxin exposure, and IL-8 protein was elevated in amniotic fluid at 72 h and at 7 days (16). The inflammatory cells recruited to the lungs were activated based on hydrogen peroxide production only at 5 and 24 h, and cell numbers were decreasing by 7 days.

The acute inflammatory responses in the fetal lung resolved quickly, and the remodeling and maturation effects then became evident. However, IL-8 remained elevated in alveolar washes and was increased at 7 days, and inflammatory cells persisted. We previously found that cells in amniotic fluid continued to express proinflammatory cytokines for 7 days after a single dose of intra-amniotic endotoxin, and repeated doses of endotoxin did not cause increased inflammation (13, 16). Therefore, the brief inflammatory response of the fetal lung may result from a downregulation of the response of the fetal lung to the inflammatory stimulus rather than disappearance of the stimulus.

In the fetal lung, the alveolar type II cells are not fully developed, and SP-B is only partially processed (1, 10). In these animals, more cells stained for TTF-1 than for SP-B, indicating that some of the immature type II cells were not making enough SP-B to immunostain. We found that the immature alveolar type II cells that contained SP-B responded to intra-amniotic endotoxin with loss of SP-B. In adult mice, the secretion of SP-B after intratracheal adenovirus instillation was a marker of alveolar type II cell injury (29). Two events related to SP-B occurred after intra-amniotic endotoxin. At 5 h, the lung injury caused a loss of
mature SP-B from alveolar type II cells, followed by a lung maturation response with large increases in SP-B in the tissue and the alveoli at 7 days. Therefore, the initial injury response of the immature alveolar type II cells was followed by a maturation of the alveolar type II cells.

The transient decrease of alveolar type II cells at 24 h coincided with the maximal percent of apoptotic cells. Alveolar type II cells can undergo apoptosis in vivo after injury (2). Because the lung parenchyma-to-air space volume ratio changes after intra-amniotic endotoxin exposure, lung structure and cell numbers also change at each time point. The quantification of alveolar type II cells relative to all cells is independent of the thinning of the parenchyma and should give a reasonable estimate of alveolar type II cell numbers. The general conclusion was that alveolar type II cells did not change much because of a balance between cell loss and proliferation.

We have better characterized the early phase of lung injury that occurs in our model of endotoxin-induced chorioamnionitis. The chorioamnion and the fetal lung are inflamed very soon after exposure to intra-amniotic endotoxin, and the inflammation persists for several days. Elements characteristic of lung injury in adult animals occurred in the preterm lung soon after endotoxin exposure. There was a sequence of injury and inflammatory changes in the fetal lung within hours of endotoxin exposure that was followed by apoptotic cell death and remodeling within days. The injury resolves with thinning of the mesenchyme, increases in surfactant lipids and proteins, and an arrest of septation (14–16, 26). These changes appear physiologically to be maturation because lung mechanics and gas exchange improve (13). However, the resolution of injury resulted in altered alveolar development, also characteristic of oxidant- or ventilation-induced lung injury in the preterm (4, 5, 23). We recently reported that IL-1 given by intra-amniotic injection can also induce the same alterations in lung development that result from endotoxin exposure (27). A question that remains to be resolved is whether injury to the fetal lung invariably triggers alterations in subsequent lung development.

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