BASIC SCIENCE: OBSTETRICS

Human amnion epithelial cells reduce ventilation-induced preterm lung injury in fetal sheep

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OBJECTIVE: The objective of the study was to explore whether human amnion epithelial cells (hAECs) can mitigate ventilation-induced lung injury.

STUDY DESIGN: An established in utero ovine model of ventilation-induced lung injury was used. At day 110 of gestation, singleton fetal lambs either had sham in utero ventilation (IUV) (n = 4), 12 hours of IUV alone (n = 4), or 12 hours of IUV and hAEC administration (n = 5). The primary outcome, structural lung injury, was assessed 1 week later.

RESULTS: Compared with sham controls, IUV alone was associated with significant lung injury: increased collagen ($P = .03$), elastin ($P = .02$), fibrosis ($P = .02$), and reduced secondary-septal crests ($P = .009$). This effect of IUV was significantly mitigated by the administration of hAECs: less collagen ($P = .03$), elastin ($P = .04$), fibrosis ($P = .02$), normalized secondary-septal crests ($P = .02$). The hAECs were immunolocalized within the fetal lung and had differentiated into type I and II alveolar cells.

CONCLUSION: The hAECs mitigate ventilation-induced lung injury and differentiated into alveolar cells in vivo.

Key words: amnion epithelial cells, bronchopulmonary dysplasia, fetal sheep, ventilation-induced lung injury

Cite this article as: Hodges RJ, Jenkin G, Hooper SB, et al. Human amnion epithelial cells reduce ventilation-induced preterm lung injury in fetal sheep. Am J Obstet Gynecol 2012;206:448.e8-15.

With advances in perinatal care, survival rates for very preterm babies have greatly increased.[1] However, with this increased survival has come new morbidities reflecting the developmental and functional challenges faced by the extreme preterm infant.

In particular, bronchopulmonary dysplasia (BPD), or neonatal chronic lung disease, affects about 1 in 3 very preterm babies.[1] In various combinations, mechanical ventilation, oxygen toxicity, and inflammation can damage the structurally immature lung and derail normal lung development, resulting in arrested alveolarization, disorganized vascular formation, increased interstitial cellularity, and fibrosis, the hallmarks of BPD.[2,3] Even antenatal corticosteroids, given to accelerate lung maturation, reduce acute respiratory distress syndrome and increase survival, stall alveolarization, and contribute to the development of BPD.[4] Whatever the cause(s), babies that develop BPD sustain long-term impairment in their lung function, often requiring domiciliary oxygen therapy, and have associated increased risks of neurodevelopmental impairment.[1]

Currently, there is no effective treatment for BPD. We and others have recently shown that human amnion epithelial cells (hAECs), cells derived from term human fetal membranes, can prevent and repair acute adult lung injury.[5-8] In this study, using a large animal model of ventilation induced lung injury (VILI) that induces BPD-like changes in lung histology,[9] we investigated whether hAECs could ameliorate preterm lung injury.

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Received Dec. 7, 2011; revised Feb. 8, 2012; accepted Feb. 29, 2012.

This study was supported by project grant 606438 from the National Health and Medical Research Council of Australia, a Glynn White Research Scholarship (R.J.H.) from the RANZCOG Research Foundation, and the Victorian Government's Operational Infrastructure Support Program.

The authors report no conflict of interest.

Presented at the 57th annual meeting of the Society for Gynecologic Investigation, 2010. R.J.H. was awarded the President's Award for Young Investigators.

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0002-9378/$36.00
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doi: 10.1016/j.ajog.2012.02.038

MATERIALS AND METHODS

Isolation and preparation of hAECs

Placentae were collected from women with normal healthy singleton pregnancies undergoing elective cesarean section between 37 and 42 weeks' gestation, in accordance with Southern Health Human Research Ethics Committee approval. Human amnion epithelial cells were derived as previously described in detail.[8] Briefly, amnion was peeled from the chorion, rinsed in phosphate-buffered, and subjected to an initial 10 minutes digest with 0.25% trypsin at 37°C with agitation, discarding the first cells. The remaining amnion was then subjected to 2 more 1-hour trypsin digests, deactivating trypsin with one-tenth volume of newborn calf serum. The cell isolates from the 2 1-hour digests were pooled, counted using a hemocytometer, and resuspended at 15 million cells/mL. Cells were stained with 5 μM carboxyfluorescein succinimidyl ester (CFSE) and

resuspended in sterile phosphate-buffered saline (PBS; 30 million cells per 2.5 mL).

Animal experiments

Animal experimental protocols were approved by the School of Biomedical Science, Monash University Animal Ethics Committee. Pregnant Merino × Border Leicester ewes at 105 days gestational age were anesthetized and underwent surgery. (Term in sheep is approximately 147 days. Day 105 is equivalent to a human gestation of about 26 weeks.) The fetal head was delivered at hysterotomy (while anesthetized), and heparinized polyvinyl vascular catheters were inserted into the carotid artery, jugular vein, and trachea. A modified endotracheal tube was secured in the lower trachea and connected to 2 large-bore, saline-filled ventilation tubes (inner diameter 9.5 mm, outer diameter 14.3 mm). A further saline-filled catheter (inner diameter 3.2 mm, outer diameter 6.4 mm) was inserted into the upper trachea and connected to 1 of the ventilation tubes to facilitate normal flow of lung liquid through this exteriorized tracheal loop, thereby preventing tracheal occlusion and potential lung growth and maturation.[9] All catheters and tubing were exteriorized via the ewe's flank. The fetus remained in situ, and the maternal abdomen was closed. We allowed 5 days of recovery and monitored fetal arterial blood gas results daily to ensure well-being.

At gestational age (GA) 110 days (saccular stage of fetal lung development), we disconnected the ventilation tubing to the upper tracheal catheter and drained the lung liquid into a sterile bag. We then connected the ventilation tubing to a neonatal ventilator (Draeger 8000+; Draeger, Lübeck, Germany). Fetuses were ventilated with room air for 12 hours using a peak inspiratory pressure of 40 cm H_2O, a positive end-expiratory pressure of 4 cm H_2O, an inspiratory flow of 15 L/min, and a rate of 65 breaths/min. This ventilation regimen has been shown to cause lung injury including inflammation and histological changes similar to BPD.[9,10]

Lung liquid was replaced at the end of ventilation. Of the animals receiving ventilation, 4 animals received in utero ventilation (IUV) alone, and 5 animals received IUV and hAECs (IUV plus hAECs). In this latter group, 30 million hAECs, in 2.5 mL of PBS, were administered each to the fetal jugular vein and tracheal catheter over 5 minutes at both 3 and 6 hours into the IUV (total 120 million hAECs).

These administration time points were chosen to allow sufficient time for a fetal inflammatory response to occur but with a focus on proof-of-principle prevention rather than treatment of established disease. The dose of hAECs given was scaled up from previous experiments in mice that had proven efficacy of the cells.[6,7] The total dose was split into 2 to minimize volume to the fetus. Four control animals did not receive IUV.

Tissue analysis of lung injury

The ewe and fetus were humanely killed with sodium pentobarbitone overdose at GA 117 days' gestation, 7 days after the IUV. We chose the ventilation-to-outcome interval of 7 days based on our previous experience in which significant lung injury was evident 1 week following brief in utero ventilation.[9]

Prior to the collection of fetal lungs, bronchoalveolar lavage (BAL) fluid was drained via the tracheal tube and collected for subsequent cell analyses. Fetal lungs were processed for histology as previously described.[9] Briefly, the lungs were removed, the left bronchus ligated with random portions of the left lung used for fluorescence-activated cell sorting (FACS) analysis or snap frozen, and stored at −80°C. The right lung was fixed at 20 cm H_2O with 4% paraformaldehyde via the trachea, postfixed in Zamboni's fixative, and processed for light microscopy. The right lung was separated into each lobe and cut into 5 mm slices. Three pieces from each lobe were randomly selected using a random number table and cut into 2 cm^2 sections (5 mm thick) and embedded in paraffin. Blocks were then randomly selected from each lobe and cut at 5 μm, incubated at 60°C (2 hours), deparaffinized, rehydrated, and washed in PBS.

Sections were then stained with hematoxylin and eosin, Hart's resorcinfuschin stain to identify elastin, and Gordon and Sweet reticulum stain to identify collagen I and III. Immunohistochemistry was used to identify myofibroblasts (alpha smooth muscle actin [αSMA]). Tissue/airspace ratio and secondary septal crest densities were measured by image analysis using a point-counting technique using 3 sections and 5 fields per view.[9] Elastin, collagen, and αSMA-staining density were measured by image analysis (ImagePro plus; MediaCybernetics, Bethesda, MD) using 3 sections with 5 random fields per view. All analyses were performed by a single observer (R.J.H.) blinded to the experimental groups. Five random samples of lung were analyzed for the presence of CFSE positive cells by flow cytometry using Mo-Flo XDP Cell Sorter (Beckman Coulter, Gladesville, Australia) and Summit version 5.0 software (Dako, Campbellfield, Australia).

αSMA immunohistochemistry

Five-micrometer sections were deparaffinized and rehydrated. Antigen retrieval was performed in citrate buffer. Endogenous peroxidases were quenched, and nonspecific binding was blocked with 20% normal goat serum for 60 minutes at room temperature. Sections were incubated with primary antibody (antihuman αSMA; DakoCytomation, Glostrup, Denmark) for 90 min (room temperature), washed in PBS (with 0.1% Tween-20) for 5 minutes (×3), and incubated with biotinylated secondary antibody (antimouse Biotinylated Ab; Vector Laboratories, Burlingame, CA) in PBS for 1 hour. Sections were washed in PBS/0.1% Tween 20 for 5 minutes (×3) and the biotinylated secondary antibody detected (Vectastain ABC; Vector Laboratories). Sections were washed, dehydrated, mounted, and viewed using light microscopy.

Immunolocalization of hAECs

To investigate possible mechanisms underlying any effect of hAEC administration, we explored whether hAECs had engrafted within the fetal lung epithelium and differentiated into lung alveolar epithelial cells[11,12] and whether there was modulation of the host inflammatory response.

Sections were deparaffinized, rehydrated, boiled in sodium citrate as above, incubated with blocking buffer (4% normal goat serum; 0.2% Triton X-100),

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