BASIC SCIENCE: OBSTETRICS

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

Ryan J. Hodges, MBBS (Hons); Graham Jenkin, PhD; Stuart B. Hooper, PhD; Beth Allison, PhD; Rebecca Lim, PhD; Hayley Dickinson, PhD; Suzie L. Miller, PhD; Patricia Vosdoganes, PhD; Euan M. Wallace, MD

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


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.

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 saline, 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. 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 (sacular 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 H2O, a positive end-expiratory pressure of 4 cm H2O, 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.

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. 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.

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. 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 H2O 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 cm² 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 resorcinfuscin 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/air-space ratio and secondary septal crest densities were measured by image analysis using a point-counting technique using 3 sections and 5 fields per view.

**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 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),
and washed in PBS (×3). Sections were incubated overnight with rabbit antihuman prosurfactant protein C (1:1000; Chemicon International, Temecula, CA) at 4°C. Sections were incubated at room temperature for 2 hours with the secondary antibodies goat α-fluorescein 488 (1: 200; Invitrogen, Carlsbad, CA) and donkey α-rabbit 568 (1:1000; Invitrogen). Sections were washed in PBS and stained with 4′,6-diamidino-2-phenylindole nuclear stain for 5 minutes, mounted with fluorescent mounting media (Dako, Glostrup, Denmark), and imaged with a confocal microscope (Nikon CI upright; Nikon, Melville, NY).

To examine whether hAECs engrafted within the fetal lung epithelium and differentiated into lung alveolar epithelial cells, we used FACS for CFSE-labeled cells and analyzed representative samples of lung homogenate and BAL for each fetus that received IUV plus hAECs. We also performed FACS analysis on tissue homogenates from the fetal brain, thymus, heart, liver, spleen, adrenals, kidneys, and mesenteric lymph nodes. Specifically, tissue samples (approximately 100 mg) were minced and subjected to 70 μm filtration to generate a single-cell suspension for subsequent analysis (BD FACS Canto II Flow; BD Biosciences, North Ryde, New South Wales, Australia). Gates were optimized for exclusion of dead cells and autofluorescence. CFSE-positive cells are expressed as a percentage of total cells per sample analyzed.

**Quantitative real-time polymerase chain reaction (PCR)**

We measured messenger ribonucleic acid (mRNA) of interleukin (IL)-1, IL-6, IL-8, surfactant protein (SP)-A, SP-B, transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) from lung tissue using real-time quantitative polymerase chain reaction. Primer sequences used, gene accession numbers, and regions amplified for SP-A, SP-B, SP-C, VEGF, and PDGF are shown in the Table.

<table>
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<th>Gene</th>
<th>GenBank accession #</th>
<th>Nucleotides amplified</th>
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<th>Downstream primer 5’-3’</th>
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<td>GCCCATTGGTAGAAGAACCC</td>
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PCR, polymerase chain reaction; PDGF-A, platelet-derived growth factor; SP, surfactant protein; VEGF, vascular endothelial growth factor.


**Statistical analysis**

Values are expressed as mean ± SEM. Comparisons between groups were made using a 1-way analysis of variance, followed by Bonferroni’s multiple comparison post hoc test. A value of $P < .05$ was considered statistically significant. Analyses were performed with GraphPad Prism software version 5.0b (GraphPad Software, Inc, La Jolla, CA, http://www.graphpad.com).

**RESULTS**

Twelve hours of IUV alone produced many of the histological features of BPD. Specifically, compared with controls, IUV alone increased the proportion of tissue collagen within the lung (mean ± SEM 22.9 ± 2.5% vs 10.3 ± 1.1%, $P = .03$), elastin content (mean ± SEM 15.9 ± 2.4% vs 4.5 ± 1.2%, $P = .02$), and the tissue/airspace ratio (mean ± SEM 1.56 ± 0.47 vs 0.23 ± 0.03, $P = .04$) and reduced the number of developing alveoli as assessed by secondary septal crest number (mean ±...
SEM 2.6 ± 0.3% vs 11.5 ± 1.1%, P = .009) (Figure 1).

The administration of hAECs during IUV significantly mitigated the ventilation-induced lung damage. Compared with IUV alone, hAECs reduced collagen (9.9 ± 0.7%, P = .03) and elastin deposition (7.8 ± 1.2%, P = .04), reduced the tissue/airspace ratio (0.73 ± 0.24, P = .1), and preserved the proportion secondary septal crest number (8.7 ± 0.7%, P = .02) (Figure 1).

We recovered mean ± SEM 0.05 ± 0.02% of whole-lung tissue that was positive for live CFSE-labeled cells and 0.23 ± 0.1% from the BAL. CFSE-labeled cells were undetectable in all other organs examined (fetal brain, thy-
whether the increased TGFβ1 expression, administration of hAECs was associated with significantly less αSMA staining than that seen in IUV alone animals, to a level near control animals (17.4 ± 0.9% P = .02) with a normal spatial pattern of elastin immunostaining (Figure 4). Although there was a trend toward reductions in other profibrotic mediators in IUV-alone fetuses compared with those ventilated animals that received hAECs, these were not significantly different: VEGF (162.8 ± 101 vs 0.8 ± 0.6 P = .09) and PDGF (5.2 ± 0.3 vs 0.6 ± 0.4 P = .4) (Figure 3).

Treatment with hAECs also resulted in a reduction in the fetal inflammatory response compared with IUV alone. In particular, IL-8 expression was increased 20-fold in the IUV-alone group (21.8 ± 5.6 P = .02) and significantly inhibited to near control levels by hAECs (0.11 ± 0.05 P = .04) (Figure 3). Although not statistically significant, we also observed a trend in fetuses in the IUV-alone group vs hAECs to reduced IL-1 (111 ± 74.7 vs 0.85 ± 0.8, IUV alone vs IUV plus hAECs; P = .1), IL-6 (69.2 ± 44.5 vs 0.05 ± 0.03; P = .1), and surfactant gene expression, a known response system leading to the inflammation of BPD15; SP-A (12.5 ± 6.8 vs 0.24 ± 0.2 P = .05), SP-B (51 ± 32.5 vs 0.3 ± 0.2 P = .1), and SP-C (3.9 ± 2.1 vs 0.14 ± 0.1 P = .07) (Figure 3).

**COMMENT**

Advances in perinatal care have significantly increased survival rates for very preterm babies but at the expense of increased morbidity. This has brought an urgent need to develop novel preventive strategies to minimize the burden of chronic disease attributed to the very interventions essential for their survival. Our observations in this study may offer one such approach. Specifically, we have shown that hAECs are capable of preventing VILI, reducing elastin and collagen deposition and preserving both septal crest number and the spatial pattern of elastin deposition. In short, maintaining the normal lung architecture required for ongoing alveolar development and thereby, hopefully, preventing BPD. We believe that these findings represent a significant advancement over the...
current management of BPD, which is largely supportive.\textsuperscript{16}

The majority of previous studies using hAECs in lung injury have addressed their potential use in small animal models of adult lung disease, using injurious insults such as bleomycin or naphthalene.\textsuperscript{5-7} In all these models, hAECs were able to reduce lung injury and to some extent, at least in immune compromised mice, integrate into the lung epithelium. More recently we have shown that hAECs are also able to mitigate infection mediated fetal lung injury in an in utero model of chorioamnionitis.\textsuperscript{17}

Similarly, others have explored the use of mesenchymal stem cells (MSCs) as a treatment for hyperoxia-induced neonatal lung injury.\textsuperscript{18,19} What is clear from all of these studies, including the current study, is that hAECs and MSCs do offer promise as a cell therapy for acute lung injury, whether in the fetus, the newborn, or the adult.

What remains much less certain though is how these cells might effect their reparative actions. A recurrent observation, confirmed in this current study, is that there is a very low rate of cell engraftment. This suggests that, despite their established in

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**FIGURE 3**

Fetal inflammatory response

TGF-β\textsubscript{1}, VEGF, PDGF, IL-1, IL-6, IL-8, SP-A, SP-B, and SP-C mRNA levels (mean ± SEM) in fetuses with no IUV (control), IUV alone, and IUV plus hAECs. Values are expressed as a fold change relative to values in unventilated control fetuses (no IUV). Asterisk indicates $P < .05$.

hAEC, human amnion epithelial cells; IUV, in utero ventilation; PDGF, platelet-derived growth factor; TGF-β\textsubscript{1}, transforming growth factor beta; VEGF, vascular endothelial growth factor.

vitro pluripotent ability to differentiate into all cell lineages. hAECs are unlikely to primarily effect their reparative actions via in vivo cellular integration and differentiation. Indeed, in the current study, that only 0.05% of lung cells were hAECs would suggest that engraftment is not likely to be clinically relevant.

Consistent with this, 2 recent studies in immune competent adult mice, prevention of acute lung injury by hAECs was achieved without any apparent integration of hAECs into the lung epithelium. Rather, it would appear that modulation of the host response to injury by hAECs is the more likely mechanism. If this is correct, then identifying the antifibrotic and anti-inflammatory pathways involved in lung repair will better direct the targeted use of cell therapeutics, such as hAECs. In this regard, experiments in cornestal injury indicate that amniotic membrane is anti-inflammatory, and similarly we have shown, first in a mouse model of adult lung injury, that hAECs are able to mitigate the host inflammatory response to lung. The current study has afforded, we believe, some further insights into how these cells may work.

Specifically, ventilation is thought to cause BPD by triggering such an inflammatory response that promotes fibrosis and abnormal elastin deposition. TGFβ, in particular, is regarded as a key regulator of fibroblast migration and differentiation into the myofibroblast phenotype, leading to excessive collagen and elastin deposition in a functionally abnormal spatial pattern away from the tips of developing septal crests. The significant reductions in TGFβ gene expression coupled with normal spatial αSMA staining after administration of hAECs in the study support that hAECs may be modulating myofibroblast differentiation, either directly or indirectly. This is consistent with reports of down-regulation of TGFβ receptors and reversal of fibroblast-myofibroblast differentiation of ocular fibroblasts when cultured on amniotic membrane. Furthermore, the reduction in IL-8 following hAEC administration is likely to be fundamental to the cells’ ability to suppress a proinflammatory milieu.

IL-8 is a potent attractant for neutrophils and macrophages and thereby functions to maintain the pulmonary inflammatory response and subsequent fibrosis and scarring. In the setting of newborn lung disease, increased levels of IL-8 in plasma and tracheobronchial aspirate in neonates are predictive of subsequent BPD. In the adult mouse models of lung injury, hAECs have been shown to decrease IL-8 in the lung and to decrease neutrophil and macrophage influx. Although we were unable to specifically address the effects on lung macrophages in this current study, an effect on macrophages either directly or indirectly via IL-8 would be consistent with the decreased TGFβ and αSMA that we observed.

Our animal model of VILI has some important limitations that merit consideration. The in utero ventilation does not attempt to mimic the clinical setting, in which obviously no baby is ventilated in utero. Rather, it mimics the acute lung injury, and subsequent lung histology, of BPD and allows assessment of injury and repair at a time point remote from the insult. This is necessary if we are to explore the mechanisms underlying such repair but avoids the practical limitations of ventilating a preterm lamb ex utero long enough to assess injury and repair over weeks. Naturally, even in the absence of hAECs, spontaneous repair of VILI occurs in utero, as occurs with bleomycin injury in adult models, emphasizing the need for appropriate control animals, as described here. Furthermore, we did not administer glucocorticoids ahead of ventilation and hAEC administration.

Currently, it would be expected that the majority of preterm infants that will require ventilation would have received antenatal glucocorticoids in preparation for their birth. It is feasible that the maturational effects that glucocorticoids have on the preterm lung also change the lungs’ responses to injury. It would certainly be worthwhile examining whether the effects of hAECs in this model of injury are maintained following glucocorticoid priming.

It is also important to note that we were able to study only a small number of animals. Such a small number precluded the ability to assess whether the...
reparative effects of hAECs would be a consistent outcome across many animals or, when applied clinically, neonates.

Moving forward, hAECs have a number of properties that suggest they are likely to translate well into clinical therapy. They are a noncontroversial source of cells, are relatively easily collected and isolated, have stable telomere length without evidence of tumour formation, and exhibit low antigenicity. In short, hAECs offer the promise of a readily accessible autologous, and possibly allogeneic, cell therapy for preterm infants to prevent BPD, thereby conferring placental protection well into the newborn period.

This study provides the rationale for further research to determine the optimum dose, timing, and route of administration of hAECs required to minimize BPD. In that regard, we have recently described cell isolation and preparation methodologies that are animal product (eg, calf serum) free and that would be compliant with good manufacturing practice, a necessity for clinical cell therapies. Furthermore, the potential interaction with other common clinical therapies such as antenatal glucocorticoids and postnatal surfactant therapy warrants evaluation.

REFERENCES