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Preterm human amnion epithelial cells have limited reparative potential

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ABSTRACT

The collection and use of stem cells from the fetal membranes as cell therapy for a variety of lung diseases, including preterm lung disease, have been previously proposed. To date, only cells from term amnion have been assessed. In the setting of a future therapy for the preterm neonate, it would be ideal if autologous cells could be given. However, the reparative and anti-inflammatory actions of stem cells isolated from preterm amnions have not been evaluated. In this study, with a view to developing an autologous cell therapy for preterm lung injury, we compared the differentiation potential and efficacy of term versus preterm human amnion epithelial cells (hAECs) to protect against inflammation and fibrosis in a bleomycin mouse model of lung injury. We found that, unlike term hAECs, preterm hAECs did not differentiate into a lung lineage following culture in small airway growth media. Preterm hAECs also exerted significantly less protective effects than term hAEC following acute lung injury. Specifically, preterm hAEC did not improve Ashcroft scoring or collagen deposition in the lung despite a reduction in activated myofibroblasts. Term hAECs expressed double the levels of HLA-G compared to preterm hAECs. These findings indicate that while hAECs can be isolated from term and preterm amnions in similar numbers, they bear distinctive characteristics, which may impact upon their clinical use.

1. Introduction

Preterm infants, particularly those born very or extremely preterm, face significantly increased risks of morbidity and mortality. In this regard, there has been increasing interest in the use of stem cells as therapies for various disease specific to the preterm new born including bronchopulmonary dysplasia (BPD), necrotizing enterocolitis and hypoxic–ischemic encephalopathy. Over recent years, we have undertaken a series of studies evaluating the utility of human amnion epithelial cells (hAECs) in lung repair with a view to developing an autologous source of cells as a therapy for BPD. We commenced this work having shown that hAECs, derived from term placental membranes, were able to differentiate down multiple cell lineages, including into alveolar epithelial cells, both *in vitro* and *in vivo* [1]. We, and others, have shown that hAECs are able to both prevent [2–4] and repair [5] bleomycin-induced lung injury in adult mice, reducing inflammation and fibrosis to normalize lung architecture and improve lung function [4]. Subsequently, we demonstrated that hAECs are able to prevent fetal lung injury in two sheep models of BPD, namely lipopolysaccharide (LPS)-induced chorioamnionitis [6] and ventilation induced lung injury [7]. In both of these models of BPD, hAECs given at the time of the injury reduced pulmonary inflammation and normalized lung architecture including increased septal crest density and tissue:airspace ratio.

While the results from studies to date suggest that hAECs may be a promising cell therapy for BPD, the preventative or reparative efficacy of preterm hAEC has not been assessed. This is important because the development of an autologous cell therapy will depend on the efficacy of preterm hAECs since BPD is a disease of the extreme preterm baby. Accordingly, in this current study we set out to assess the differentiation capacity of preterm hAECs and their ability to mitigate lung damage in a mouse model of lung injury.

2. Materials and methods

2.1. Isolation of hAECs

Placentae were obtained from women undergoing caesarean sections. The mean (range) gestational age at collection for preterm births was 30 (26–34) weeks, and for term births 38 (37–39) weeks as detailed in Table 1. The indications for preterm births included discordant twin growth, preeclampsia and intrauterine growth restriction (IUGR). Exclusion criteria included chorioamnionitis, active labor or pre-existing maternal disease including diabetes. The term caesarean sections were electives in otherwise healthy women. All women provided written informed

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Table 1
Characteristics of volunteer subjects and isolated term and preterm hAECs.

Plurality	Gestational age	Cell yield (millions)	Viability immediately after isolation (%)	Post-thaw (%)	Complications
Term twins	37 (+4)	110.0	88.0	78.6	IUGR
		81.0	89.0	82.5	
	38 (+5)	79.4	74.3	65.4	IUGR
		49.6	68.0	52.9	
Term singletons	37 (+1)	74.0	78.0	77.0	None
	38 (+5)	69.0	80.9	80.2	None
	38 (+3)	101.3	86.7	86.4	None
	37 (+0)	97.0	87.0	87.0	None
Preterm twins	32 (+2)	246.5	89.2	79.5	IUGR
		70.0	78.6	77.4	
	36 (+4)	110.0	91.1	88.0	
		81.0	89.0	90.0	IUGR
	34 (+6)	189.0	89.5	82.6	
		131.3	89.0	85.4	IUGR
Preterm singletons	26 (+3)	20.0	89.0	86.7	IUGR
	25 (+3)	83.9	89.4	88.0	PE
	29 (+5)	128.8	88.0	86.6	PE
	30 (+0)	219.0	93.0	92.6	PE
	31 (+5)	182.0	91.70	91.2	PE

Gestational age is indicated as week (+days); IUGR: intrauterine growth restriction; PE: preeclampsia.

consent. The collection of placentae was undertaken with the approval from the Southern Health Human Research Ethics Committee.

Preterm hAECs were isolated as previously described for term amnion [8]. Briefly, the amnion was stripped from adjacent choriodecidua and rinsed in Hanks Balanced Salt Solution (HBSS; Invitrogen, San Diego, CA). hAECs were isolated by two consecutive digests in 0.05% trypsin (Invitrogen). Total cell yield and viability were determined by cell count and Trypan blue exclusion. All hAECs, both preterm and term, were kept frozen and tested for purity of population by FACS analysis against epithelial cell adhesion molecule (EpCAM) (BD Bioscience, 347198), CD90 (BD Pharmingen, 559869) and CD105 (BD Pharmingen, 555690). Microbial contamination was also tested prior to animal administration by 7-day cell culture in the absence of antibiotics. Only non-contaminated cells with a purity of >96% EpCAM + cells and viability >85% were used. Purity of hAEC isolates was determined based on the criteria of EpCAM+CD90−CD105− by FACS analysis.

2.2. Differentiation of hAECs in vitro

In vitro differentiation of term and preterm hAECs towards lung epithelial lineage was performed as previously described [8] where mRNA transcription for mature alveolar cells (surfactant proteins C and D; SPC, SPD), adipocytes (lipoprotein lipase; LPL) and chondrocytes (osteonectin and osteocalcin) was determined by RT-PCR. Cells were thawed and allowed to recover in DMEM/F12 media for 3–5 days before culturing in small airway growth medium (SAGM; Lonza, Walkersville, MD, USA), adipogenic or osteogenic media, as previously described [8].

2.3. Proliferation assay

Proliferation of term and preterm hAECs was investigated using a colorimetric MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) according to manufacturer's instruction. Term and preterm hAECs were plated separately at a seeding density of 8000 cells per well in a 96-well plate. Twenty microliters of MTS substrate was added to each well at day 3, 7 and 11 and plates were returned to their incubators for 4 h prior to absorbance readings at 490 nm (SpectraMax, Molecular Devices, Sunnyvale, CA).

2.4. Animals and experimental groups

All animal experiments were approved by the Monash Medical Centre Animal Ethics Committee and conducted according to the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (2006). Eight to ten week old female C57/BL6 mice weighing 16–20 g were housed in a specific pathogen free animal facility for the duration of this study. Experimental groups included saline + saline, saline + term hAECs, bleomycin + saline group, bleomycin + term hAECs and bleomycin + preterm hAECs; $n = 8$ per group. Briefly, 0.3 U bleomycin (Blenoxane, Hospira, IL, USA) was administered intranasally [4] followed by intraperitoneal administration of term hAECs, preterm hAECs or vehicle (saline) 24 h later. Four million hAECs were resuspended in 200 μl in sterile saline for intraperitoneal injection. Mice were culled either 7 or 14 days following intranasal instillation of bleomycin.

2.5. Harvesting of mouse tissues

Mice were culled by carbon dioxide asphyxiation. The right lungs were inflated to 25 cmH_20 and fixed with 4% (*w*/*v*) paraformaldehyde processed for histological analysis. The left lungs were ligated at the main bronchus, excised and cut into small pieces before snap freezing. Pieces of left lung tissue were randomly allocated for molecular analysis.

2.6. Histological examinations

Paraffin sections were cut at 5 μm thickness and stained with hematoxylin and eosin (H&E) Ashcroft scoring for inflammation and fibrosis as previously described [9]. Briefly, 20–25 non-overlapping sequential fields of view (200× magnification) were independently examined by three scorers (RL, STC, JLT) blinded to the treatment groups. An overall mean (±SEM) score was calculated.

Collagen content was determined morphometrically as previously described [4,10]. Briefly, area of tissue staining positive for collagen was quantified using Metamorph analysis software v7.7 (Molecular Devices, Sunnyvale, CA, USA). For each histological section, the entire lung was imaged using bright field microscopy at 200× magnification but excluding blood vessels (BX41, Olympus American Inc., Centre Valley, PA, USA). The area positive for collagen staining was expressed as a percentage of total tissue area.

2.7. Assessment of macrophage number

Citrate antigen retrieval was performed on 5 μm paraffin sections before application of primary antibodies against macrophage marker, F4/80 (1:100, AbD Serotec, Düsseldorf, Germany). Sections were incubated with biotinylated goat anti-rat secondary antibody (1:200, Chemicon, Billerica, MA, USA), EnVision detection system (LSAB+ System HRP, Dako, Glostrup, Denmark) and liquid DAB substrate (Dako). Tissues were counterstained with hematoxylin. Lungs were imaged at 200× magnification, excluding blood vessels, where 10 non-overlapping fields were captured and analyzed. The percentage area positive for F4/80 cells was quantified using Metamorp v7.7 (Molecular Devices).

2.8. Western blotting for α smooth muscle actin and HLA-G

Total lung protein (40 μg) was loaded per well. Western blots were performed using mouse monoclonal anti-α smooth muscle actin (αSMA) antibody (1:500; sc-53142, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-human leukocyte antigen-G (HLA-G; 1:5000; ab52455, Abcam, Cambridge, MA, USA) or anti-β-actin antibody (1:5000; sc-47778, Santa Cruz Biotechnology Inc.). Goat anti-mouse secondary antibodies were used (1:20,000; sc-2005, Santa Cruz Biotechnology Inc.). Protein expression was determined using a chemiluminescence detection kit (Supersignal West Pico, Thermo Fisher Scientific, Scoresby, VIC, Australia).

2.9. Data analyses

Data for each animal experiment group was expressed as mean ± standard error of mean (SEM). Statistical significance was attributed where $p < 0.05$. One-way ANOVA and post-hoc Bonferroni were used to determine statistical differences between multiple groups. All analyses were performed using Graphpad Prism V5.0 (Graphpad Software Inc., San Diego, CA, USA).

3. Results

3.1. Yield and viability of preterm hAECs are comparable to term hAECs

hAECs were isolated from 8 term amnions and 11 preterm amnions as detailed in Table 1. The average cell yield of hAECs from preterm amnions was significantly higher compared to term (132.9 ± 20.9 versus 82.7 ± 6.9, $p = 0.0315$). Viability immediately after isolation was significantly greater in preterm hAECs but post-thaw viability was similar between term and preterm hAECs (Fig. 1a). Preterm hAECs were significantly more proliferative than term hAECs, where proliferation rates were double that of term hAECs by day 7, as determined by MTS proliferation assay (Fig. 1b, $p < 0.05$). Preterm hAECs did not express lung lineage specific genes SPC and SPD following culture in SAGM (Fig. 1c) but did express markers of adipogenic and osteogenic differentiation (Fig. 1d). The purity of hAECs was comparable between term and