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Effect of epithelial stem cell transplantation on noise-induced hearing loss in adult mice

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ABSTRACT

Noise trauma in mammals can result in damage to multiple epithelial cochlear cell types, producing permanent hearing loss. Here we investigate whether epithelial stem cell transplantation can ameliorate noise-induced hearing loss in mice. Epithelial stem/progenitor cells isolated from adult mouse tongue displayed extensive proliferation in vitro as well as positive immunolabelling for the epithelial stem cell marker p63. To examine the functional effects of cochlear transplantation of these cells, mice were exposed to noise trauma and the cells were transplanted via a lateral wall cochleostomy 2 days post-trauma. Changes in auditory function were assessed by determining auditory brainstem response (ABR) threshold shifts 4 weeks after stem cell transplantation or sham surgery. Stem/progenitor cell transplantation resulted in a significantly reduced permanent ABR threshold shift for click stimuli compared to sham-injected mice, as corroborated using two distinct analyses. Cell fate analyses revealed stem/progenitor cell survival and integration into suprastrial regions of the spiral ligament. These results suggest that transplantation of adult epithelial stem/progenitor cells can attenuate the ototoxic effects of noise trauma in a mammalian model of noise-induced hearing loss.

Introduction

Hearing loss due to noise overexposure is one of the most common sensory disabilities in humans, particularly in industrialised countries. This debilitating disease significantly reduces quality of life by negatively impacting upon communication in social and professional settings. The ætiology of noise-induced hearing loss (NIHL) is multifactorial involving a complex interplay between environmental and genetic factors (Ohlemiller, 2008; Konings et al., 2009). Acute noise trauma results in mechanical damage, enhanced mitochondrial free radical formation, and reduced cochlear blood flow (Henderson et al., 2006; Le Prell et al., 2007). The sequelae of these changes are extensive and include necrosis, apoptosis and sublethal pathologies in tissues throughout the cochlea (McFadden et al., 2005; Ohlemiller, 2008). Stem cell transplantation is rapidly gaining interest as a potential therapy to prevent or reverse this cell loss and thereby provide a treatment for NIHL (Martinez-Monedero and Edge, 2007; Parker et al., 2007; Revoltella et al., 2008; Brigande and Heller, 2009).

The effects of noise trauma include both transient and persistent increases in hearing threshold levels (temporary and permanent threshold shifts, respectively) (Nordmann et al., 2000; White et al., 2009). Susceptibility to the permanent effects of noise exposure differs markedly between individuals in humans and animal models of NIHL with respect to both the extent of hearing loss and the cochlear tissues affected (human: Taylor et al., 1965; Pawelczyk et al., 2009; animal: Erway et al., 1996; Davis et al., 2001; Ohlemiller and Gagnon, 2007). The CBA/Ca inbred mouse strain family has proven to be an invaluable model for the study of the pathology and treatment of NIHL as their hearing levels remain stable with age (Hunter and Willott, 1987; Schone et al., 1991; Jimenez et al., 1999), thus eliminating conflicting contributions of presbycusis.

Several studies have identified the cellular targets of noise trauma in CBA/Ca mice. Depending upon the degree of trauma, these can include the cochlear lateral wall (fibrocytes of the spiral ligament, and marginal, intermediate and basal cells of the stria vascularis), the organ of Corti (hair cells and supporting cells), and the spiral limbus (Wang et al., 2002; Hirose and Liberman, 2003; Ohlemiller and Gagnon, 2007). As several of these cochlear cell types are epithelial in origin (e.g., hair cells, supporting cells, marginal cell layer of the stria vascularis), we postulated that epithelial stem/progenitor cell transplantation could possess the potential to ameliorate NIHL. Transplanted stem cells can repair tissues by replacing damaged

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cells or by secreting factors that enhance the survival and/or proliferation of endogenous cells (Bernardo et al., 2009; da Silva Meirelles et al., 2009; Lai et al., 2010).

We and others have demonstrated that the epithelium of the tongue represents an accessible and abundant source of adult stem and progenitor cells (Ookura et al., 2002; Luo et al., 2009; Okubo et al., 2009; Sullivan et al., 2010). Adult stem/progenitor cells have a number of advantages for cochlear transplantation in that they can be used for autologous transplantation (to resist host rejection) and are less tumourigenic than embryonic stem cells (Bithell and Williams, 2005). Tissue homeostasis in adult epithelia is maintained by stem cells residing in the basal-most cell layer that give rise to progenitor cells which proliferate for a finite number of times generating several epithelial and taste bud cell types (Jones et al., 1995; Lavker and Sun, 2000; Fuchs and Horsley, 2008; Blanpain and Fuchs, 2009; Haegebarth and Clevers, 2009).

In the present study, we isolated adult stem/progenitor cells from CBA/CaH mouse tongue epithelium and characterised their proliferative capacity and phenotypes in vitro. Subsequently, we examined the efficacy of cochlear transplantation of these cells in reducing noise ototoxicity. Epithelial stem/progenitor cells were transplanted into the cochleae of CBA/CaH mice shortly after noise trauma (48 h) and hearing levels were then measured after 4 weeks. Survival and incorporation of the transplanted cells were also investigated by cell fate analyses. Together, the results of these studies provide evidence that epithelial stem/progenitor cell transplantation can engender a functional rescue of hearing in an animal model of NIHL.

Materials and methods

All procedures were approved by the Garvan Institute of Medical Research/St Vincent's Hospital Animal Ethics Committee and conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004) of the National Health and Medical Research Council of Australia. Every effort was taken to minimise discomfort to the animals.

Epithelial stem/progenitor cell isolation and culture

CBA/CaH mice (6 weeks; $n=5$) were anaesthetised with CO_2 and decapitated. The tongue was dissected free and injected with a solution of 2 mg/ml collagenase D (Roche). Following 90 min, the dorsal epithelium at the rear of the tongue (surrounding and including the circumvallate papilla) was peeled off the underlying muscle with fine forceps. This region was selected as the circumvallate papilla represents a readily identifiable landmark, thus enabling the same tissue to be isolated across animals. Tissues were minced with fine scissors and incubated in TrypLE Express (Invitrogen) containing 1 mg/ml collagenase D and 1 mg/ml hyaluronidase (Sigma) at 37 °C for 1 h. Dissociated cells were cultured in Advanced DMEM/F12 medium containing 20 mM glutamine, 10% fetal bovine serum, B-27 supplement minus vitamin A, 20 ng/ml EGF, 20 ng/ml bFGF, 100 U/ml penicillin G and 100 μg/ml streptomycin on plastic tissue culture dishes coated with rat-tail collagen (5 μg/cm^2; Roche) at 37 °C with 5% CO_2. Cells at passage 6 were used for transplantation experiments.

Immunocytochemistry

Cells were grown on glass coverslips coated with rat-tail collagen (5 μg/cm^2; Roche) and fixed at confluency for 10 min in methanol at −20 °C (for cytokeratin 8 and cytokeratin 14 immunolabelling) or 4% paraformaldehyde in 0.1 M phosphate-buffered saline pH 7.4 (PBS; for p63 immunolabelling) at 4 °C. Cells were then blocked for 1 h in 10% normal goat serum in PBS containing 0.3% Triton X-100 (NS-PBSTx). Primary antibodies were diluted in NS-PBSTx and applied for 2 h at room temperature. The following primary antibodies were used: monoclonal mouse anti-p63 (1:50; Santa Cruz Biotechnology; sc-8431), monoclonal rat anti-cytokeratin 8 (1:20; Developmental Studies Hybridoma Bank; TROMA1), monoclonal mouse anti-cytokeratin 14 (1:50; Chemicon; CBL197), monoclonal rat anti-5′-bromo-2′-deoxyuridine (BrdU; 1:250; AbD Serotec; MCA2060) and monoclonal mouse anti-BrdU (1:20; Developmental Studies Hybridoma Bank; G3G4). Cells were then rinsed for 4 h in several changes of PBS and incubated for 1 h at room temperature in the appropriate secondary antibodies diluted in PBS. Fluorescent secondary antibodies used were as follows: Alexa 488-conjugated goat anti-mouse IgG (1:100; Invitrogen; A-11029) and DyLight 649-conjugated goat anti-rat IgG (1:100; Jackson ImmunoResearch; 112-495-167). Sections were counterstained by incubation overnight at 4 °C in rhodamine-conjugated *Phaseolus vulgaris* leucoagglutinin (PHAL; 1:100; Vector Laboratories; RL-1112), fluorescein-conjugated Jacalin (1:100; Vector Laboratories; FL-1151), or the nuclear stain DAPI (0.3 μM; Invitrogen). In experiments examining mitotic activity, BrdU (Sigma) was added to the culture medium at a final concentration of 10 μM 2 h prior to fixation. The fixed cells were then incubated in 2 N HCl at room temperature for 20 min. Following rinsing in 0.1% Triton X-100 in PBS for 20 min, cells were processed for BrdU immunolabelling as described above.

Noise trauma and hearing threshold detection

CBA/CaH mice (male and female; 4–6 weeks; $n=11$) were deafened in both ears by noise overexposure (120 dB SPL, 1–80 kHz broadband noise, 2–2.5 h under general anaesthesia) in a foam-padded, shielded acoustic chamber. This strain of mice was selected to match the stem/progenitor cell donors and minimise immunorejection. Animals of this age were selected as vulnerability to noise exposure declines after 8 weeks of age in CBA/CaJ mice (Kujawa and Liberman, 2006).

Auditory function was assessed by measuring auditory brainstem response (ABR) thresholds to click and pure tone stimuli, as described previously (Bogaerts et al., 2008). Briefly, acoustic stimuli were delivered to anaesthetised mice via an electrostatic insert speaker (Tucker Davis Technologies) fitted into the external ear canal. Clicks and pure tone bursts (20 kHz) were delivered and ABRs were recorded while sound intensity was reduced in 5 dB SPL steps beginning at 90 dB SPL. ABR thresholds were determined by identifying the lowest sound intensity level at which the peak amplitude of the evoked ABR signal exceeded four times the standard deviation of the baseline noise (Bogaerts et al., 2009).

To assess the extent of NIHL, permanent ABR threshold shifts were determined by comparing the pre-trauma threshold levels in the operated (left) ear to threshold levels in the non-operated (right) ear 30 days post-trauma (Miller et al., 1963). Animals that did not display a permanent threshold shift (i.e., shift<10 dB SPL) were excluded from study.

Stem/progenitor cell transplantation

Prior to transplantation, isolated adult epithelial stem/progenitor cells were grown in flasks (Corning) to 70–80% confluency and then harvested using TrypLE Express. The collected cells were rinsed in DMEM/F12 (Invitrogen), centrifuged for 5 min at 300 × *g*, resuspended in PBS at 2000–4000 cells/μl, and stored on ice until transplanted.

To investigate the functional effects of stem/progenitor cell transplantation, mice with equivalent hearing levels at 2 days post-trauma were divided into two cohorts: Transplant and Sham. The Transplant cohort received a unilateral cochlear injection of epithelial stem/progenitor cells ($n=7$) and the Sham cohort received a unilateral injection of the vehicle solution alone ($n=4$). Cochleostomies were performed in the lateral wall of the left cochlea at the basal turn, posterior to the stapedial artery and in line with the round window as described previously (Bogaerts et al., 2008). This cochleostomy site corresponds to the 51.4 ± 2.8 kHz ($n=3$) region

of the mouse cochlea according to the place-frequency map of Müller et al. (2005). It is important to note, however, that the place-frequency map of the mouse cochlea can shift by up to one octave following noise damage (Müller and Smolders, 2005). This cochleostomy site has been shown to deliver transplanted cells primarily to the two perilymphatic compartments, scala vestibuli and scala tympani (Bogaerts et al., 2008). For stem/progenitor cell transplantations, 1 µl of cells suspended in PBS was injected over 1 min to transplant 2000–4000 cells. The cochleostomy was then sealed with bone wax, with all surgeries completed in 30–40 min.

Cell fate analyses of transplanted adult epithelial stem/progenitor cells

To investigate the fate of transplanted cells in the mouse cochlea, stem/progenitor cells (prepared as above) were labelled with the lipophilic dye Vybrant CM-DiI (5 µl/ml; Invitrogen) and injected into the cochleae of CBA/CaH mice 4–5 weeks old (10,000 cells/µl; $n=6$) using the microsurgical approach described above. Mice were sacrificed 1–4 weeks after surgery by perfusion through the left ventricle with 4% paraformaldehyde. Transplanted cochleae were removed and postfixed in the same fixative for 24 h at 4 º C. For decalcification, cochleae were exposed to 10% EDTA (Sigma) for 48 h at 4 °C. Tissues were rinsed in PBS for 1 h, cryoprotected in graded sucrose/PBS solutions to 30% sucrose (w/v), frozen in OCT (Sakura Finetek), sectioned along the modiolar axis at 7 µm on a cryostat, counterstained with NeuroTrace 500/525 green fluorescent Nissl stain (1:50; Invitrogen), and mounted in Gelmount. Sections containing transplanted cells were then immunolabelled for Na^+/K^+-ATPase expression using a rabbit monoclonal anti-Na^+/K^+-ATPase α antibody (1:100; Epitomics; 2047-1) and standard immunohistochemical methods (Sullivan et al., 2010).

To examine the distribution of transplanted cells along the cochlear axis, Vybrant CM-DiI-labelled cells were injected into the cochleae of CBA/CaH mice (4 weeks of age; 4000 cells; $n=5$) 2 days post-noise trauma. Mice were sacrificed 5 days after surgery by perfusion through the left ventricle with 4% paraformaldehyde. Transplanted cochleae were postfixed in the same fixative for 24 h at 4 º C and decalcified by exposure to 10% EDTA for 48 h at 4 °C. Surface preparations of the cochlear spiral were prepared from the apex to the base and the frequency locations of transplanted cells were determined using the place-frequency map of Müller et al. (2005).

Microscopy and image processing

Specimens were viewed using a Zeiss Axioplan epifluorescence microscope equipped with Plan-Neofluar 10 × 0.30 NA and Plan-Neofluar 20 × 0.50 NA dry objective lenses and an AxioCam MRm

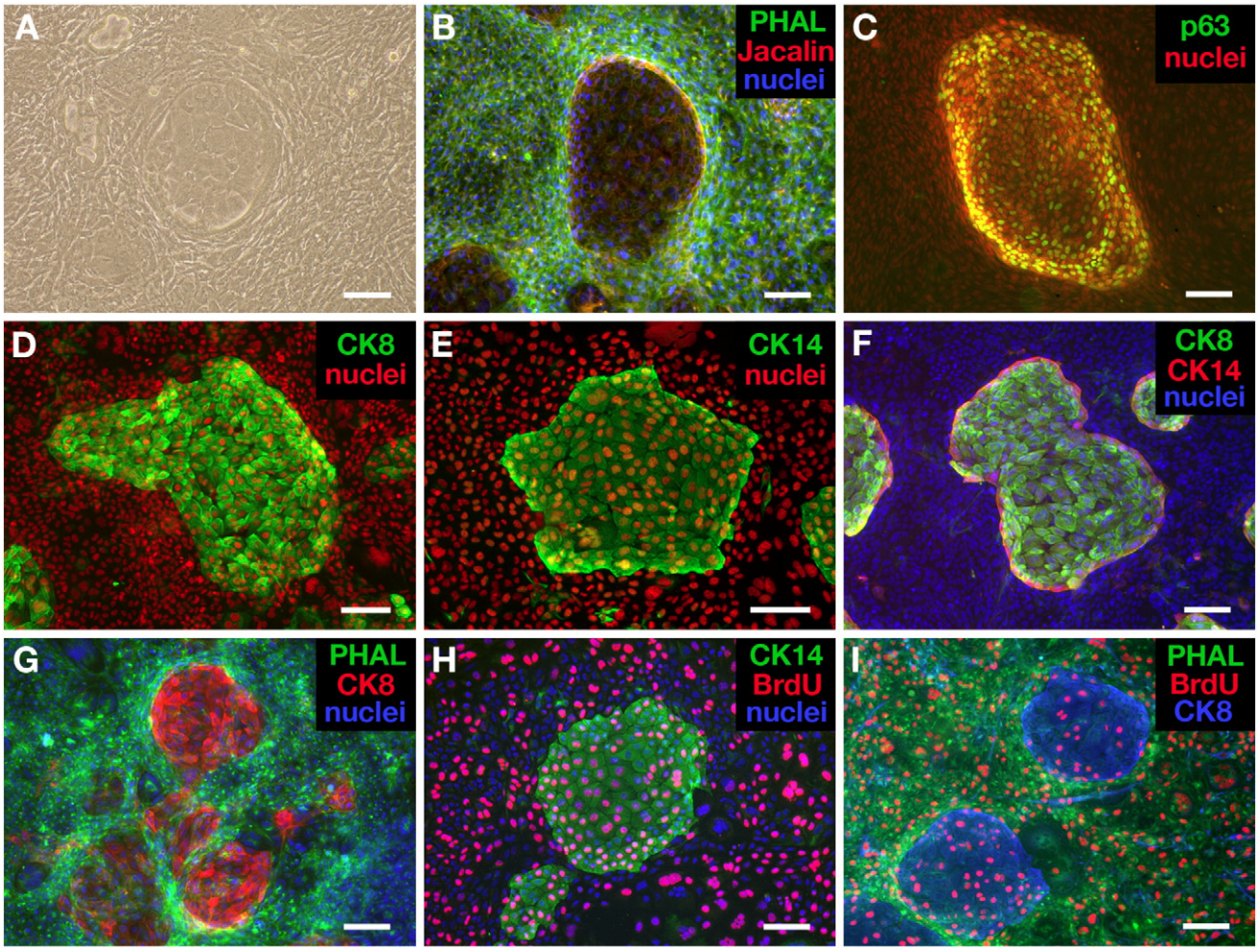

Fig. 1. Characterisation of tongue epithelial stem/progenitor cells in vitro. A. Light micrograph showing detail of a colony generated by cells isolated from the dorsal tongue epithelium. These colonies are comprised of small, densely packed cells surrounding islands of squamous cells. A representative island can be seen in the centre of the image. B. The small, densely packed cells within the colonies bind the lectins PHAL and Jacalin. C–G. Cells comprising the squamous islands express the epithelial stem cell marker p63 (C), and the epithelial markers CK8 (D, F, and G) and CK14 (E and F). H and I. Immunolabelling for BrdU incorporation showing that mitotic activity is distributed throughout the colonies, including both the populations of lectin-binding cells and the islands of squamous cells. Abbreviations: CK8, cytokeratin 8; CK14, cytokeratin 14; PHAL, *Phaseolus vulgaris* leucoagglutinin. Scale bars = 100 µm.