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Research paper

Morphological and morphometric characterization of direct transdifferentiation of support cells into hair cells in ototoxin-exposed neonatal utricular explants

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ABSTRACT

We have studied aminoglycoside-induced vestibular hair-cell renewal using long-term culture of utricular macula explants from 4-day-old rats. Explanted utricles were exposed to 1 mM of gentamicin for 48 h, during 2nd and 3rd days *in vitro* (DIV), and then recovering in unsupplemented medium. Utricles were harvested at specified time points from the 2nd through the 28th DIV. The cellular events that occurred within hair cell epithelia during the culture period were documented from serial sectioned specimens. Vestibular hair cells (HCs) and supporting cells (SCs) were systematically counted using light microscopy (LM) with the assistance of morphometric software. Ultrastructural observations were made from selected specimens with transmission electron microscopy (TEM). After 7 DIV, i.e. four days after gentamicin exposure, the density of HCs was 11% of the number of HCs observed in non-gentamicin-exposed control explants. At 28 DIV the HC density was 61% of the number of HCs observed in the control group explant specimens. Simultaneously with this increase in HCs there was a corresponding decline in the number of SCs within the epithelium. The proportion of HCs in relation to SCs increased significantly in the gentamicin-exposed explant group during the 5th to the 28th DIV period of culture. There were no significant differences in the volume estimations of the gentamicin-exposed and the control group explants during the observed period of culture. Morphological observations showed that gentamicin exposure induced extensive loss of HCs within the epithelial layer, which retained their intact apical and basal linings. At 7 to 14 DIV (i.e. 3–11 days after gentamicin exposure) a pseudostratified epithelium with multiple layers of disorganized cells was observed. At 21 DIV new HCs were observed that also possessed features resembling SCs. After 28 DIV a new luminal layer of HCs with several layers of SCs located more basally characterized the gentamicin-exposed epithelium. No mitoses were observed within the epithelial layer of any explants.

Our conclusion is that direct transdifferentiation of SCs into HCs was the only process contributing to the renewal of HCs after gentamicin exposure in these explants of vestibular inner ear epithelia obtained from the labyrinths of 4-day-old rats.

1. Introduction

Hearing and balance disorders are very common disabilities in society. One of the most frequent causes of sensorineural hearing loss and peripheral vestibular disorders is loss of the mechanoreceptive hair cells (HCs) and/or primary sensory neurons of the inner ear (Nadol, 1993; Van Eyken et al., 2007). Although deleterious

Abbreviations: AIC, Akaike's information criterion; ANOVA, analysis of variance; Atoh, atonal homologue; bHLH, basic helix-loop-helix; C group, control group; DIV, days *in vitro*; DT, direct transdifferentiation; FITC, fluorescein iso-thiocyanate; G group, gentamicin-exposed group; HC, hair cell; LM, light microscopy; P4, 4-day-old; PBS, phosphate buffered saline; QQ-plots, Gaussian reference distribution; SC, support cell; TEM, transmission electron microscopy; TF, transcription factors, TGF-a

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changes occur in adjacent neurons and at higher levels within the central nervous system following HC loss, it is generally believed that regeneration of new HCs will provide alleviation of hearing and balance impairments (Stone and Cotanche, 2007). New HCs are produced throughout life in the inner ears of amphibians and fish, and in the vestibular labyrinths of birds (Corwin, 1985; Jorgensen et al., 1988; Kil et al., 1997; Popper et al., 1984, 1990). Furthermore, in amphibians and birds HC loss induced by noise damage or ototoxic drugs can initiate the process of HC regeneration that leads to a renewal of the damaged sensory epithelia and a re-establishment of hearing and balance functions (Cruz et al., 1987; Dye et al., 1999; Jones et al., 1992; McFadden et al., 1989; Raphael, 1992; Ryals et al., 1988; Stone et al., 1998).

Regeneration of HCs in auditory and vestibular organs of non-mammalian vertebrates is known to involve both renewed mitotic activity of progenitor cells and also direct trans-differentiation (DT) of the supporting cells (SCs) remaining in the sensory epithelia after the HC loss (Adler et al., 1996; Baird et al., 2000; Corwin et al., 1988; Jones et al., 1996; Ryals et al., 1988; Taylor et al., 2005). In the avian auditory epithelium it is described that the first new HCs arising via DT appear 3–4 days after gentamicin exposure, i.e. 1–2 days earlier than the first new mitosis derived HCs are observed. However, 6 days after an ototoxic trauma the majority of the new HCs are derived from mitotic activity within the damaged sensory epithelium (Roberson et al., 2004).

For mammals, HC loss was thought to be irreversible (Meiteles et al., 1994; Ruben, 1967). Different research groups proposed that there is a limited replacement of HCs within the damaged vestibular sensory epithelia of the inner ear of mammals (Forge et al., 1998, 1993; Tanyeri et al., 1995; Warchol et al., 1993). A small, but significant, spontaneous renewal of HCs was demonstrated in the vestibular sensory epithelia of several mammalian species, *in vivo* for the guinea-pig, chinchilla and mouse, and *in vitro* for the rat, following an ototoxic injury (Berggren et al., 2003; Forge et al., 1993; Kawamoto et al., 2009; Tanyeri et al., 1995). DT of SCs into HCs without a preceding mitotic stage was shown to be the mechanism during renewal of mammalian HCs both *in vitro* and *in vivo* renewal (Cotanche, 2008; Izumikawa et al., 2005; Staecker et al., 2007). Transcription factors (TFs) participate during normal development but also have the capacity to induce DT of SCs into HCs (Schimmang, 2013). Atoh 1 is currently the only known TF expressed and acting early enough to be a decision-making gene for differentiation of HCs (Jahan et al., 2013). The expression of the Atoh 1 gene is turned off in adult mammals, but is reactivated in supporting cells after neomycin exposure in adult mouse utricles (Lin et al., 2011). Delivery of the Atoh 1 gene to the mature ototoxin-damaged inner ear of mammals induces renewal of HCs with improvement of hearing and balance (Izumikawa et al., 2005; Staecker et al., 2007). The inner ear is encased by the temporal bone, which makes repeated observations of sensory epithelia hard to obtain. In *in vitro* studies the administration of ototoxic drugs is verifiable and the concentration of the drug at the target HCs is predictable. The degree of trauma (the dosage of gentamicin) was shown to be important for the damage not only to the HCs but also to the SCs (Kawamoto et al., 2009). It has also been suggested that a trauma might cause no detectable damage to the SCs but could limit their ability to participate in transformation (Kawamoto et al., 2009). *In vitro* methods are also applicable for high throughput studies. Therefore, *in vitro* techniques are extremely useful and can be utilized with the organotypic culture of mammalian inner ear sensory epithelia providing an excellent model for investigation of HC and SC behaviour within the inner ear explants (Cunningham, 2006; Holley, 2005).

To study the cellular events that lead to the renewal of HCs our research group developed an *in vitro* model of utricular maculae explanted from four day old rats that was stable in long-term culture (i.e. up to 28 days *in vitro*), and suitable for both immunohistochemical and morphological studies (Berggren et al., 2003; Werner et al., 2012). We recently described time-dependent changes that *in vitro* conditions, by itself, have on explanted HC epithelia (Werner et al., 2012). The overall morphology of the epithelia was well preserved during culture, but there was a moderate level of cell loss. On average there was a 1.7% per days *in vitro* (DIV) decrease of the vestibular macula HC density, whereas the corresponding decrease of SC density was less than 1% per DIV. Using the same *in vitro* model, the spontaneous renewal of HCs was also demonstrated following gentamicin induced HC loss (Berggren et al., 2003). A peak of stereociliary bundle renewal occurred at three weeks post gentamicin exposure. However, in that previous study only the surface characteristics of the explanted FITC-phalloidin stained macula epithelium were studied. In the present study we use serial sectioned preparations to investigate the phenomena of DT of SCs into HCs after gentamicin exposure.

The aims of the present study were to evaluate morphological and morphometric characteristics of HCs and SCs during the process of SC to HC transdifferentiation after gentamicin exposure in cultures of 4-day old rat utricles.

2. Material and methods

2.1. Animals

Litters of 4-day-old (P4) Wistar rat pups were obtained from Charles River Laboratories. The P4 rats stayed with their mother until shortly before they were euthanized by rapid cervical dislocation. The care and use of animals was approved by the Regional Care and Use Committee of the University of Umeå (A 49-08) and conformed to NIH guidelines for the care and use of laboratory animals.

2.2. Organ culture technique

The heads of the P4 rats were sprayed with 70% ethanol. The temporal bones were removed and the capsule covering the vestibule was opened. Utricular maculae were excised as a unit and their otoconial membranes gently removed. The explanted preparations were composed of the complete macular epithelium with a minimal amount of sub-epithelial tissue. The dissection was performed in Dulbecco's phosphate buffered saline (PBS) supplemented with glucose (6 g/L) at room temperature. The explants were placed with their epithelial surfaces uppermost on perforated insert membranes (Transwell Clear with pore-size 0.4 μm. Costar, Cambridge, MA). The inserts were placed in 6-well-plates containing Dulbecco's Modified Eagles Medium (DMEM) supplemented with glucose to a final concentration of 6 g/L, 10% fetal bovine serum, N-1 supplement (Sigma) 100 μl to 10 ml of medium, and penicillin (100 U/ml). By reducing the depth of the nutrient medium above the insert membrane the explants became flattened and anchored to the culture insert membrane. The explants were incubated at 37 °C in a humidified 5% carbon dioxide atmosphere and the medium was renewed every second day.

2.3. Culture paradigms

The day of explantation was denoted as day zero *in vitro* (0 DIV). Explants were cultured for up to 28 DIV. During the initial 48 h of incubation all explants were cultured in medium without gentamicin to allow the explants to adhere to the insert membrane and