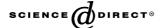
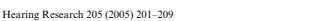


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# Disruption and restoration of cell–cell junctions in mouse vestibular epithelia following aminoglycoside treatment

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#### Abstract

The intracellular junction complexes, which consist of tight junctions (TJ), adherens junctions (AJ), and desmosomes, mediate cell-cell adhesion in epithelial cells. E-cadherin, which is a major component of AJ, plays a role not only in the maintenance of cell-cell junctions, but also in repressing cell proliferation. In this study, we examined changes of E-cadherin expression in mouse vestibular epithelia following local application of neomycin using immunohistochemistry and western blotting, and morphology of cell-cell junctions by transmission electron microscopy (TEM). Immunohistochemistry and western blotting revealed down-expression of E-cadherin and its consecutive recovery. TEM demonstrated temporal disruption of cell-cell junctions. Morphology of cell-cell junctions was more rapidly restored than recovery of E-cadherin expression. Transient disruption of cell-cell junctions and down-expression of E-cadherin is a rational response for the deletion of dying hair cells, and may be associated with a limited capacity for cell proliferations in mammalian vestibular epithelia following their rapid restoration.

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#### 1. Introduction

The intracellular junctional complexes of epithelial cells consist of three components: tight junctions (TJ),

Abbreviations: TJ, tight junction(s); AJ, adherens junction(s); NM, neomycin; TEM, transmission electron microscopy; SC, supporting cell(s); HC, hair cell(s); PSCC, posterior semicircular canal ducts; PBS, phosphate-buffered saline; PLSD, Protected Least Significant Difference; NBT, toluidine *p*-nitrotetrazolium blue; BCIP, 5-bromo-4-chloro-3-indolylphosphate; PB, phosphate buffer; HCL, hair cell layer; SCL, supporting cell layer

\* Corresponding author. Tel.: +81 75 751 3346; fax: +81 75 751 7225. E-mail addresses: kim@ent.kuhp.kyoto-u.ac.jp (T.-S. Kim), tnaka-gawa@ent.kuhp.kyoto-u.ac.jp (T. Nakagawa). adherens junctions (AJ), and desmosomes (Farquhar and Palade, 1963). E-cadherin is an extracellular calcium-dependent transmembrane adhesion molecule that mediates cell–cell interaction through AJ between epithelial cells (Takeichi, 1991). Classic cadherin molecules in AJ are transmembrane homophilic adhesion receptors that indirectly associate with the actin cytoskeleton by interacting with catenins (Tsukita et al., 1992). E-cadherin plays a role not only in the maintenance of cell–cell junctions, but also in the regulation of cell proliferation, migration, and differentiation (Gumbiner et al., 1988; Birchmeier and Behrens, 1994; Vleminckx and Kemler, 1999).

There are a number of reports on E-cadherin expression in the inner ear (Whitlon, 1993; Leonova and Raphael, 1997; Whitlon et al., 1999; Hackett et al., 2002; Kim et al., 2002; Simonneau et al., 2003; Kelley, 2003). In damaged inner ear epithelia, supporting cells (SC) surrounding the dying hair cells (HC) rapidly expand and replace the lesion to maintain the barriers that prevent the mixing of endolymph and perilymph, resulting in scar formation (Forge, 1985; Raphael and Altschuler, 1991; Meiteles and Raphael, 1994; Li and Forge, 1995; Steyger et al., 1997; Forge and Li, 2000). In the mammalian cochlea, junctional proteins play a key role in organizing scar formation after aminoglycoside-induced injury (Leonova and Raphael, 1997). In the mammalian vestibular epithelia, our preliminary study (Kim et al., 2002) has indicated that an aminoglycoside, neomycin (NM), which is a potent ototoxic drug, has the potential to transiently alter the expression of adherens junctional proteins including E-cadherin. However, details in changes of cell–cell junctions following aminoglycoside treatment still fragmentary.

We have previously developed a mouse inner ear damage model using local NM application (Nakagawa et al., 2003). This model induces apoptotic cell death of HC in both cochlear and vestibular epithelia in a well-defined manner. In this study, we examined the relationship between the morphological changes of cell-cell junctions and alterations of E-cadherin expression in damaged vestibular epithelia using this model.

#### 2. Materials and methods

#### 2.1. Animals

C57BL/6 mice aged 10-week-old (SLC, Inc., Hamamatsu, Japan) were used as experimental animals. Experimental protocols and animal care were approved by the Institute of Laboratory Animals Animal Research Committee, Graduate School of Medicine, Kyoto University.

#### 2.2. Experimental groups

In the NM-treated group (*n* = 48), neomycin solution (5 μl; 40% (w/v) in physiological saline; Wako Pure Chemical Industries Ltd, Osaka, Japan) was infused into the perilymphatic space of posterior semicircular canal ducts (PSCC) of the left ear with a microsyringe at a rate of 1 μl/min (Nakagawa et al., 2003), under general anesthesia with ketamine (100 mg/kg, i.p.; Sankyo Pharmaceutical, Tokyo, Japan) and xylazine (9 mg/kg, i.p.; Bayer Japan, Tokyo, Japan). On days 1, 3 and 5 after neomycin treatment, the mice were deeply anesthetized with ketamine and xylazine, and the temporal

bones were obtained. Specimens obtained from the right ear, non-treated side, were used as controls. Animals subjected to similar procedures and microinjected with local application of physiological saline (in place of NM) were categorized as the sham-operated group. The temporal bones (n=16) were isolated on day 3 after local application of saline, and the prepared specimens were subjected to statistical analyses for the NM-treated groups.

#### 2.3. Immunohistochemistry

Each animal was perfused intracardially with 0.01 M phosphate-buffered saline (PBS) under deep anesthesia, followed by 4% paraformaldehyde in PBS. The temporal bones were then collected and immersed in the same fixative for 4 h at 4 °C. After rinses with PBS, the utricles were dissected from the temporal bones. Eight utricles from each group were subjected to immunohistochemical analysis in whole-mounts or frozen sections. Following fixation, a portion of the specimens were immersed overnight in PBS containing 30% sucrose before being embedded in OCT compound (Tissue-Tek, Sakura, Tokyo, Japan). Sections (6-µm thickness) were then cut with a cryostat (Leica Microsystems, Tokyo, Japan), and four contiguous sections of each sample at a distance 300 µm from the edge of the vestibular epithelia were provided for histological analysis.

The samples were preincubated with 0.5% Triton X-100 for 30 min at room temperature. After rinsing with PBS, the blocking procedures for immunostaining were performed using a Mouse on Mouse Kit (Vector Laboratories, Burlingame, CA). The samples were then incubated overnight at 4 °C with anti-E-cadherin monoclonal mouse IgG antibodies (×200 dilution; detection site: intracellular lesion, Transduction Laboratories, Lexington, NY), and anti-calretinin polyclonal rabbit IgG antibody (×200 dilution; Chemicon Temecula, CA) to use as a HC marker (Dechesne et al., 1994; Zheng and Gao, 1997; Zheng et al., 1999a; Desai et al., 2005). After rinses with PBS containing 10% goat serum, the samples were incubated for 1 h at room temperature with FITC-conjugated goat antimouse IgG2a (×200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-rabbit IgG-Alexa 546 conjugated (×200 dilution; Molecular Probes, Eugene, OR). After rinses with PBS, the samples were mounted onto glass slides and coverslipped with Vectashield mounting medium (Vector Laboratories). Whole-mount specimens and sections were then viewed with a confocal laser-scanning microscope (TCS-SP2 Leica Microsystems, Tokyo, Japan). In whole-mount specimens, immunolabeling for E-cadherin was examined at 2–4 µm depth below the surface of the sensory epithelia.

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