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The influences of sphingolipid metabolites on gentamicin-induced hair cell loss of the rat cochlea

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ABSTRACT

Sphingolipid metabolites inducing ceramide, sphingosine, and sphingosine-1-phosphate (S1P) play important roles in the regulation of cell proliferation, survival, and death. Aminoglycoside antibiotics including gentamicin induce inner ear hair cell loss and sensorineural hearing loss. Apoptotic cell death is considered to play a key role in this injury. The present study was designed to investigate the possible involvement of ceramide and S1P in hair cell death due to gentamicin. In addition, the effects of other metabolites of ceramide, gangliosides GM1 (GM1) and GM3 (GM3), on gentamicin ototoxicity were also investigated. Basal turn organ of Corti explants from p3 to p5 rats were maintained in tissue culture and exposed to 20 or 35 μM gentamicin for 48 h. The effects of ceramide, S1P, GM1, and GM3 on gentamicin-induced hair cell loss were examined. Gentamicin-induced hair cell loss was increased by ceramide but was decreased by S1P. GM1 and GM3 exhibited protective effects against gentamicin-induced hair cell death at the limited concentrations. These results indicate that ceramide enhances gentamicin ototoxicity by promoting apoptotic hair cell death, and that S1P, GM1, and GM3 act as cochlear protectants. In conclusion, sphingolipid metabolites influence the apoptotic reaction of hair cells to gentamicin ototoxicity.

The sphingolipid metabolites ceramide, sphingosine, and sphingosine-1-phosphate (S1P) are known as a new class of lipid second messengers and reportedly play essential roles in the regulation of cell proliferation, survival, and death [15,19]. Ceramide and sphingosine usually inhibit cell proliferation and promote apoptosis, while ceramide-derived S1P acts against apoptosis.

Ceramide has been shown to regulate diverse cellular processes including apoptosis, cell senescence, the cell cycle, and cellular differentiation [12]. The levels of ceramide are increased by stimulating sphingomyelinase which converts sphingomyelin to ceramide. Several signaling molecules, such as tumor necrosis factor, Fas ligand, γ-interferon, interleukin 1, vitamin D, and stressful events including radiation and ischemia, can activate sphingomyelinase and promote ceramide-induced apoptosis. Ceramide activates stress-activated protein kinases (SAPKs) such as jun kinases (JNKs), kinase suppressor of Ras (KSR), and atypical protein kinase C (PKC). Ceramide also activates protein phosphatases such as protein phosphatase 1 (PP1) and protein phosphatase 2A (PPA2) [19]. However, the mechanisms of how ceramide activates protein kinases and phosphatases have never been fully elucidated. Sphingosine is formed only by the deacylation of ceramide [8]. Sphingosine has been reported to be a protein kinase C inhibitor and induce apoptosis [15]. In contrast, another metabolite of ceramide, sphingosine-1-phosphate (S1P), influences opposing pathways of apoptosis and cell survival. For example, S1P stimulates the extracellular signal-regulated kinase (ERK) pathway and counteracts SAPKs [4]. Ceramide, sphingosine, and S1P are interconvertible, and their relative levels are suggested to be important to determine cell fate. It has been shown that sphingosine kinase, the enzyme that phosphorylates sphingosine to form S1P, is a critical regulator of this "sphingolipid rheostat" [15].

Gangliosides are also metabolites of ceramide. Ganglioside GM1 (GM1) has been reported to induce the synthesis of S1P [3]. GM1 has also been considered to exhibit a neurotrophic effect. Namely, GM1 prevents the degeneration of neuronal cells [5], being beneficial in treating stroke [11], spinal cord injuries [7], and Alzheimer's disease [24]. Ganglioside GM3 (GM3) is also a metabolite of ceramide that lies upstream of ganglioside GM1 [20]. There have been many studies on the effects of these sphingolipid metabolites on apoptosis in several cell lines, but their effects on cochlear pathophysiology poorly understood.

Aminoglycoside antibiotics including gentamicin induce sensorineural hearing loss and inner ear hair cell loss. Although apoptotic cell death is considered to play a key role in cochlear injury induced by aminoglycosides, the mechanisms of aminoglycoside-induced hair cell apoptosis have not been fully elucidated. This study was designed to investigate the possible

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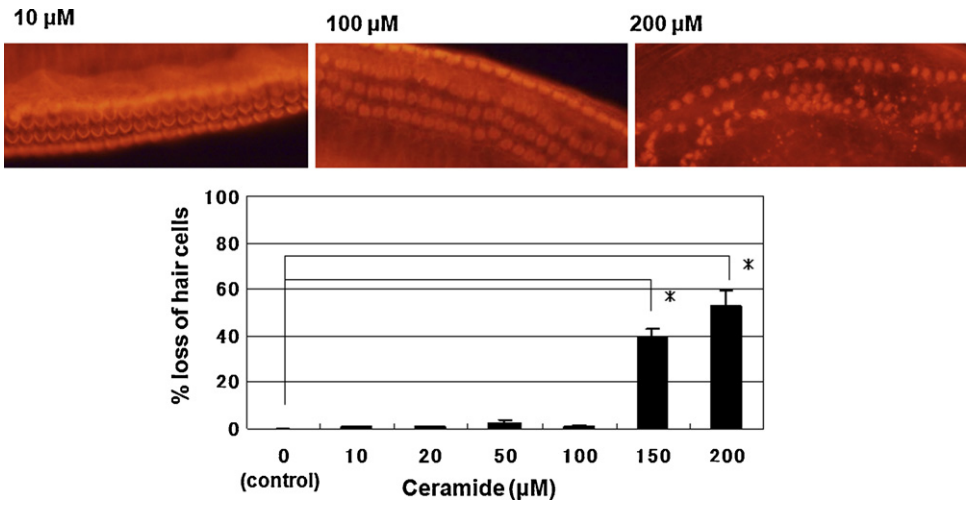

Fig. 1. Effect of ceramide on cochlear hair cells. Representative microphotographs of hair cells cultured with 10, 100, or 200 μM ceramide (without gentamicin) (upper, phalloidin staining). Quantitative analysis of hair cell loss in explants treated with ceramide (without gentamicin) for 48 h (lower). Ceramide itself induced hair cell loss at 150 and 200 μM (*one-way ANOVA and Bonferroni test: $p < 0.05$).

involvement of sphingolipid metabolites in hair cell death due to gentamicin.

The basal turn of the organ of Corti was dissected from Sprague–Dawley rats on postnatal days 3 (p3) to 5 (p5) and cultured based on the methods of Van de Water and Ruben [29] and Sobkowicz et al. [22]. All animal procedures were carried out according to guidelines of the Laboratory Animal Research Center at the University of Tsukuba.

Cochlear explants were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 25 mM HEPES, and 30 U/ml penicillin and were cultured in an incubator at 37 °C with 5% CO_2 and 95% humidity. Cochlear cultures were maintained in the above-described medium overnight (8–12 h) and then exposed to a medium containing 20 or 35 μM gentamicin for 48 h to assess the effects of sphingolipid metabolites [25,17].

All sphingolipid metabolites examined in this study were purchased from Sigma (Tokyo, Japan). The tested concentrations of ceramide, S1P, GM1, and GM3 were 10–200, 10–100, 10–1000, and 10–1000 μM, respectively. Ceramide was initially dissolved in ethanol to 15 mM and stored at −20 °C, which was diluted in the culture medium to the final concentration immediately before use. S1P was initially dissolved in methanol to 13 mM and stored at −20 °C. GM1 and GM3 were directly dissolved in the culture medium immediately before use.

After explants were cultured for 48 h in culture media containing 35 μM gentamicin alone or 35 μM gentamicin plus each concentration of sphingolipid metabolites, the explants were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and then permeabilized with 5% TritonX-100 (Sigma, St. Louis, MO, USA) in PBS with 10% fetal bovine serum (FBS) for 10 min [25,26]. The specimens were stained for phalloidin with a conjugated Alexa Fluro probe (1:100, Molecular Probes Inc., Carlsbad, CA, USA) at room temperature for 1 h. Phalloidin is a specific marker for cellular F-actin and labels the stereociliary arrays and cuticular plates of hair cells [17,25].

To identify apoptosis, the terminal deoxynucleotidyl tranferase-mediated biotinylated UTP nick-end labeling (TUNEL) procedure was applied to cultured explants using the DEAD EndTM Fluorometric TUNEL System (Promega Inc., Madison, WI, USA). At the end of culture, the explants were fixed with 4% paraformaldehyde in PBS for 20 min and then treated with blocking solution containing 5% TritonX-100 in PBS with 10% FBS for 10 min. Using the manufacturer's protocol, after rinsing in PBS and pre-equilibrating, the explants were incubated at 37 °C for 1 h in darkness in the labeling solution which contained terminal deoxynucleotidyl transferase and fluorescein nucleotides. They were then washed again in PBS. The explants were subsequently stained with an Alexa Fluro phalloidin probe to differentiate hair cells from supporting cells.

Hair cells were characterized as missing if no stereocilia and/or no cuticular plate was observed by phalloidin staining. Quantitative results were obtained by evaluating 30 outer hair cells associated with 10 inner hair cells in a given microscopic field [17,25]. The average of three separate counts was used to represent each culture.

All data are expressed as the mean ± S.E.M. Statistical analysis was performed employing unpaired *t*-tests or one-factor ANOVA followed by Bonferroni post hoc tests, as required (StatView 5.0). *p*-Values of less than 0.05 were considered significant. All experiments consisted of $n = 7–12$ explants per experimental group.

In control explants maintained in the initial medium for 48 h without exposure to ceramide, almost all hair cells including one row of inner and three rows of outer hair cells were present. The effects of ceramide and ethanol as a solvent on hair cells were examined and compared with the control. There was no significant hair cell loss when explants were cultured for 48 h in the medium containing 10–100 μM ceramide without gentamicin (Fig. 1). However, 150 and 200 μM ceramide significantly induced hair cell loss (one-factor ANOVA and Bonferroni test: $p < 0.05$ in the 150 and 200 μM subgroups as compared with the control group). Ethanol alone did not induce hair cell loss.

The effect of ceramide on hair cell loss induced by gentamicin of 20 μM (Fig. 2(A)) or 35 μM (Fig. 2(B)) was examined. Ten or 20 μM ceramide did not affect hair cell loss induced by gentamicin. However, hair cell loss significantly increased in the presence of 50 and 100 μM ceramide, although ceramide per se did not have any effect at these concentrations, as shown in Fig. 1.

In TUNEL staining, apoptotic cells were labeled if they had shrunken and fragmented nuclei. Explants exposed to 35 μM gentamicin were labeled by TUNEL staining (Fig. 3). The number of TUNEL-positive cells significantly increased in the specimens cultured with 35 μM gentamicin plus 50 μM ceramide, compared with the gentamicin control group (unpaired *t*-test: $p < 0.05$). Ceramide treatment enhanced the apoptosis of outer hair cells induced by gentamicin.

The effect of S1P on gentamicin-induced hair cell damage was examined. S1P itself did not induce hair cell loss without gentam-