**Protective effects of exogenous GM-1 ganglioside on acoustic injury of the mouse cochlea**

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**Abstract**

GM-1 ganglioside (GM-1), a glycosphingolipid, is embedded in the lipid layer of neuronal membranes and is one of the neuroprotective agents. To the best of our knowledge, the role of GM-1 has never been examined in hair cell injury. The purpose of this study was therefore to evaluate the effects of GM-1 on acoustic injury of the cochlea. Mice were exposed to 4-kHz pure tone of 128 dB SPL (sound pressure level) for 4 hours. GM-1 was intraperitoneally administered immediately before the onset of acoustic overexposure. The threshold shift of the auditory brainstem response (ABR) and hair cell loss were then evaluated 2 weeks after acoustic overexposure. Immunostaining for 4-hydroxynonenal (4-HNE), indicative of lipid peroxidation, was also examined in animals subjected to acoustic overexposure. GM-1 treatment significantly decreased the ABR threshold shifts and hair cell loss after acoustic overexposure. And immunostaining for 4-HNE was reduced by GM-1 treatment. These findings suggest that GM-1 is involved in the protection of the cochlea against acoustic injury through inhibiting lipid

peroxidation.

GM-1 ganglioside (GM-1), a glycosphingolipid with an attached monosialic acid moiety, is found in high concentrations embedded in the external lipid layer of neuronal membranes [29, 31]. GM-1 is known to exist in clusters and form microdomains, known as lipid rafts [13, 26]. GM-1 is considered to modulate various protein kinase activities [37], $Ca^{2+}$ flux [11] and neurite outgrowth [21]. In addition, GM-1 and other brain gangliosides possess antioxidant activity, significantly reducing the accumulation of lipid peroxide products and free radical production. Because of its neuroprotective and neurorestorative properties, GM-1 ganglioside has been clinically administered such as those with spinal cord injury and Alzheimer's disease [1, 2, 8, 31].

Exposure to high sound pressure levels causes hearing loss by damaging sensory hair cells of the cochlea e.g. [33]. Many reports have demonstrated that the progression of acoustic injury is advanced by oxidative stress [10, 25, 35, 42]. Endogenous antioxidants such as glutathione [41], superoxide dismutase [16], and alpha-tocopherol [15] protect the inner ear by

reducing the generation of free radicals.

The presence of GM-1 in the cochlea has been demonstrated by Santi et al [23]. We hypothesized that GM-1 protects the cochlea against acoustic injury by reducing free radicals. In this study, we examined the effects of GM-1 ganglioside on acoustic injury of the cochlea.

Seventy-six female ddY mice, 8 weeks of age, were purchased from Japan SLC (Hamamatsu, Japan). The ddY mouse is frequently used as a subject for pharmacological and toxicological experiments in Japan. The care and use of animals was approved by the Animal Experiment Committee of the University of Tsukuba.

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). Positive, negative, and ground electrodes were inserted subcutaneously at the vertex, mastoid, and back, respectively. Bursts of pure tone (rise and fall times; 1 ms, duration; 10 ms, repetition rate; 20/s in an open field system) were used to evoke the ABR. Evoked responses were filtered with a band pass of 200 to 3,000 Hz and averaged over 1,000

sweeps using a signal processor (Synax 1200, NEC, Tokyo, Japan). The sound intensity varied in 5-dB steps. The ABR was measured at three frequencies (4, 8, and 16 kHz) before, immediately after and two weeks after acoustic overexposure. ABR threshold shifts from pre-exposure levels were then examined two weeks after acoustic overexposure.

The mice were exposed to a 4-kHz pure tone of 128 dB SPL for 4 hours through an open field system inside a sound-exposure chamber (Type 4212, Brüel & Kjaer, Copenhagen, Denmark), in which two small cages (4x3x6 cm) were placed [17, 36]. Two mice were subjected at the same time.

The mice were sacrificed under deep anesthesia two weeks after acoustic overexposure. Cardiac perfusion was performed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Cochleae were quickly removed, immersed in the same fixative at 4°C for 8 hours, and then decalcified with ethylenediaminetetraacetic acid for one week. After decalcification, cochleae were dissected as surface preparations, and the nuclei of hair cells were stained with propidium

iodide (PI, 2 μg/ml in PBS, Molecular Probes Inc., OR, U.S.A.) in darkness.

The number of missing hair cells (absence of PI staining) was counted under a laser confocal microscope (TCS SP2, Leica Microsystems, Wetzlar, Germany) around the 66% region from the apex of the cochlea. Our previous reports clarified that acoustic overexposure to 128 dB SPL for 4 hours of mice induced hearing loss and the maximum hair cell loss at the 66% region from the apex of the cochlea [17, 36].

Immunocytochemical analyses were carried out on cryostat sections. The methods for the fixation and decalcification of cochleae were the same as those described in the above section. Cryostat sections were then made parallel to the modiolus to identify the organ of Corti on microscope slides. The cryostat sections of 6 μm were incubated in 0.5% Triton X-100 and blocked in calf serum for 10 min at room temperature. The sections were then washed with PBS, followed by incubation with the primary antibody at a concentration of 1:100 (anti-4HNE) (Abcam, MA, U.S.A.) at 4°C for 72 hours. The sections were then incubated with the secondary

antibody at a concentration of 1:200 (anti-goat IgG conjugated with FITC) (Abcam, MA, U.S.A.) and with PI (2 μg/ml in PBS) at room temperature for 30 min in darkness. Immunolabeling was visualized using a laser microscope (BX51-DP71-SET, OLYMPUS, Tokyo, Japan). The density level of immunofluorolabeling of outer hair cells (5 samples of each groups) was assessed with the freely available image analysis software program ImageJ (National Institutes of Health, Bethesda, MD, U.S.A.).

GM-1 was purchased from Wako (Japan), and dissolved in physiological saline solution. GM-1 and saline were administered immediately before the onset of acoustic overexposure. Mice were randomly assigned to one of the following 4 treatment groups:

1) 1 mg/kg GM-1-treated group (n=6)

2) 10 mg/kg GM-1-treated group (n=6)

3) 30 mg/kg GM-1-treated group (n=6)

4) Noise-alone group (n=6)

Immunostaining for 4-HNE before acoustic overexposure without any drugs (n=4) was examined. And immunostaining for 4-HNE of the Noise-alone group (n=4) and the GM-1 (30 mg/kg) treatment group (n=4) were compared at each following time points: 0 hours, 4 hours, 12 hours, 1 day, 3 days and 7 days after acoustic overexposure.

All data are expressed as the mean ± S.D. The comparison of ABR threshold shifts or hair cell loss between each group was performed by one-way and two-way analysis of variance (ANOVA), and then the Scheffé test and Fisher's PLSD test were used. Comparison of the densitometry on immunofluorolabeling with 4-HNE was performed using Student's t-test. A p-value of less than 0.05 was considered significant.

Figures 1 demonstrates ABR threshold shifts 2 weeks after acoustic overexposure, respectively. The GM-1 (1 mg/kg) and GM-1 (10 mg/kg) groups did not significantly reduce ABR threshold shifts (two-way ANOVA, Scheffé test and Fisher's PLSD test, $p>0.05$). On the other hand, the GM-1 (30 mg/kg) group showed a significantly decreased ABR threshold shift

(two-way ANOVA, Scheffé test and Fisher's PLSD test, $p<0.01$).

Figure 2 demonstrates representative photographs of hair cell loss at the 66% region from the apex of the cochlea in the noise-alone and GM-1 (30 mg/kg) groups. The quantitative analysis of hair cell loss is shown in Figure 3. As expected from the ABR studies, 30 mg/kg GM-1 significantly ameliorated outer hair cell loss, especially in the first row, as compared with the noise-alone group (two-way ANOVA; $p<0.05$, one-way ANOVA; $p<0.05$ in the first row, Fig. 3).

4-HNE was broadly stained in the sensory epithelium (organ of Corti) of both groups at all time points [Fig. 4A]. Slight staining was observed even before acoustic overexposure [Fig. 4A]. In the noise-alone group, staining with 4-HNE gradually increased until 12 hours after acoustic overexposure, and staining was clearly observed at 12 hours to 3 days after. The staining then became weak at 7 days after [Fig. 4A]. The time course of 4-HNE staining in the GM-1 (30 mg/kg) group was essentially similar to that of the noise-alone group. The highest density was

seen at 12 hours to 3 days after [Fig. 4B]. On the other hand, when comparing the staining at each time point, there was tendency for staining in the GM-1 (30 mg/kg) group to be weaker than that of the noise-alone group [Fig. 5]. Figure 5 shows the relative densities of 4-HNE staining in the outer hair cells subtracted from the pre-exposure level. Staining differences between both groups reached significance from 4 hours to 3 days (Student's t-test: *$p<0.05$) [Fig. 5]. However, the difference in density was not clear at 7 days [Fig. 4A].

GM-1 administered before acoustic overexposure significantly decreased ABR threshold shifts and hair cell loss at 2 weeks after acoustic overexposure. The present findings clearly demonstrated that GM-1 ganglioside ameliorated the permanent threshold shifts induced by acoustic overexposure. To our knowledge, this is the first report that GM-1 ganglioside has protective effects on hair cells against cochlear injury. Previous studies have shown the protective effect of GM-1 on the spiral ganglion and cochlear nucleus [20, 38].

It has been demonstrated that acoustic injury damages the cochlea by producing free

radicals. Many reports have demonstrated that antioxidants and free radical scavengers exhibit protective effects against acoustic injury, e.g., glutathione [41], superoxide dismutase [16], methylprednisolone [32, 34], vitamin A, C, or E [15], and tempol [17]. Acoustic overexposure initially damages the outer hair cells, and these agents effectively prevented the injury of outer hair cells. Our study is consistent with these previous studies.

In this study, we evaluated the staining of 4-HNE in the organ of Corti. 4-HNE is a toxic aldehyde commonly used as an indicator of lipid peroxidation. Oxidative stress within a cell generates reactive oxygen species that interact with phospholipids in the cell membrane to cause their peroxidation [9]. 4-HNE is produced in the cochlea damaged by sound-induced trauma, and has been shown to cause the apoptosis of auditory neurons in vitro [24, 40]. Triamcinolone acetonide and glutathione were two agents shown to protect hair cells from hydroxynonenal-induced cell death [9, 22]. GM-1 treatment decreased 4-HNE staining of outer hair cells in this study. This finding indicated that GM-1 protected outer hair cells through

inhibiting the toxic effects of free radicals and hydroxynonenals.

GM-1 was reported to have the neuroprotective effect on the cytotoxic action of hydrogen peroxide in PC12 cells which were sensitive to oxidative stress [29, 43]. GM-1 prevented the accumulation of malondialdehyde (MDA), a marker of oxidative stress, and the inactivation of $Na^+$-$K^+$-ATPase induced by hydrogen peroxide in PC12 cells. GM-1 prevented the oxidative inactivation of $Na^+$-$K^+$-ATPase induced by glutaric acid and pentylenetetrazole [7]. GM-1 was reported to decrease the glutamate-induced activation of free radical reactions in nerve cells, and also reduced neuronal death in a rat cerebellar granule cell culture [3, 4]. These studies also revealed the antioxidant properties of ganglioside, and thus supported our present findings.

GM-1 is abundantly embedded in the lipid layer of neuronal membranes. Recent studies suggest that GM-1 can function as a primary lipid messenger on the cell membrane responding to marked stress. GM-1 is inserted into the plasma membrane as the hydrophobic ceramide portion

of the molecule becomes embedded in the outer leaflet of the plasma membrane and its carbohydrate portion extends into the extracellular space [28]. This ceramide portion forms a domain receiving many external signals. Many biochemical studies reported that domains transmitted signals from the extracellular space to cytosol. These lipid rafts are associated with a variety of signaling molecules, such as GPI-anchored proteins [19], non-receptor and receptor tyrosine kinases [39], Src-family tyrosine kinases [12], and trimeric GTP-binding proteins [5] on the inside of the cell membrane. They may control mitogen-activated protein kinase (MAPK) activities, S6 kinase activities [37], the phosphorylation of Trk [18], and $Ca^{2+}$ flux [11]. Thus, GM-1 may be a kind of potentiator or modulator of trophic factors in both the peripheral and central nervous systems [6, 14, 27, 28, 30]. Further studies are necessary to clarify the involvement of the protective mechanisms of GM-1 other than its antioxidative property.

In conclusion, we showed herein that GM-1 is able to protect the cochlea against acoustic injury. The immunocytochemistry of hair cells revealed that the antioxidant property of

GM-1 was important for the protection.

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Legends

Fig. 1. ABR threshold shifts 2 weeks after acoustic overexposure.

GM-1 ganglioside significantly decreased ABR threshold shifts 2 weeks after acoustic overexposure at only 30 mg/kg (two-way ANOVA and Scheffé test: *$p<0.05$).

Fig. 2. A) Representative microscopic fluorescence image of hair cell nuclei in the noise-alone group 2 weeks after acoustic overexposure. B) Representative image of the 30 mg/kg GM-1 ganglioside group. Arrows indicate hair cell loss.

The 30 mg/kg GM-1 ganglioside group was less hair cell loss than noise-alone group.

Fig. 3. The effect of 30mg/kg GM-1 ganglioside on hair cell loss after acoustic overexposure.

Missing hair cells at the 66% region from the apex were calculated 2 weeks after acoustic overexposure. Treatment with GM-1 significantly decreased loss of OHCs, especially in the first row (two-way ANOVA: *$p<0.05$, one-way ANOVA: * $p<0.05$ in the first row).

IHC: inner hair cell, OHC1: the first row of outer hair cells,

OHC2: the second row of outer hair cells, OHC3: the third row of outer hair cells

Fig. 4. Representative photographs of 4-HNE immunostaining of the organ of Corti.

A) Photographs of the noise-alone group before and after overexposure.

B) Representative images 3 days after acoustic overexposure of the noise-alone and GM-1 (30 mg/kg) groups.

Fig. 5. Densities of 4-HNE in the outer hair cells.

Relative values subtracted from the pre-exposure level are shown. The time-course of the immunostaining density in the GM-1 (30 mg/kg) group was similar to that of the noise-alone group. The highest density was seen at 12 hours to 3 days after overexposure. Each density of immunostaining in the GM-1 (30 mg/kg) group was significantly weaker than that of the noise-alone group at the same time points from 4 hours to 3 days (Student's t-test: *$p<0.05$).