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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 h. 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 200–3000 Hz and averaged over 1000 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 2 weeks after acoustic overexposure. ABR threshold shifts from pre-exposure levels were then examined 2 weeks after acoustic overexposure.

The mice were exposed to a 4-kHz pure tone of 128 dB SPL for 4 h through an open field system inside a sound-exposure chamber (Type 4212, Brüel & Kjaer, Copenhagen, Denmark), in which two small cages (4 cm × 3 cm × 6 cm) were placed [17,36]. Two mice were subjected at the same time.

The mice were sacrificed under deep anesthesia 2 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 h, and then decalcified with ethylenediaminetetraacetic acid for

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1 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 h 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 h. 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). Control incubations were routinely processed without primary antibody. The density level of immunofluorolabeling of three outer hair cells was assessed in each cochlea 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 h, 4 h, 12 h, 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.

Fig. 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, $p < 0.01$).

Fig. 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 Fig. 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).

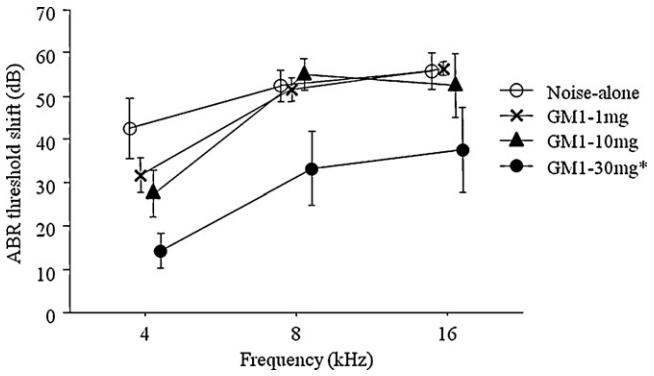

Fig. 1. ABR threshold shifts 2 weeks after acoustic overexposure. GM-1 ganglioside significantly decreased ABR threshold shifts 2 weeks after acoustic overexposure at only 30 mg/kg (two-way ANOVA and Scheffé test: $^*p < 0.05$).

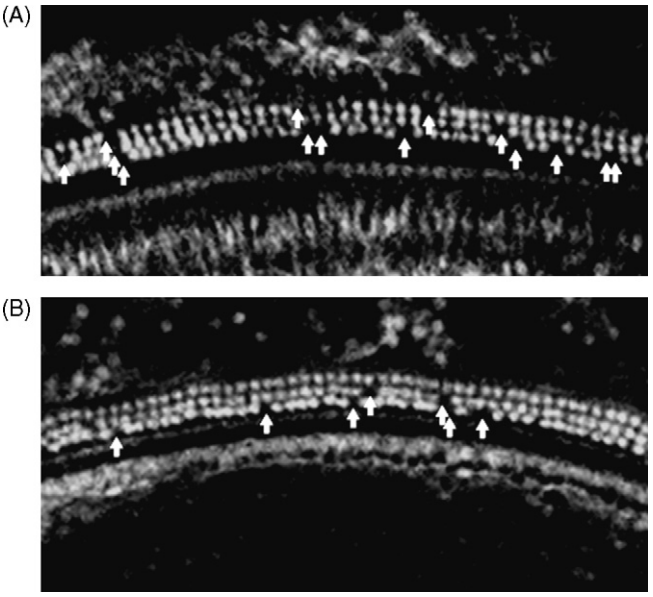

Fig. 2. (A) Representative microscopic fluorescence image of hair cell nuclei in the noise-alone group 2 weeks after acoustic overexposure. (B) Representative image of the 30 mg/kg GM-1 ganglioside group. Arrows indicate hair cell loss. The 30 mg/kg GM-1 ganglioside group was less hair cell loss than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

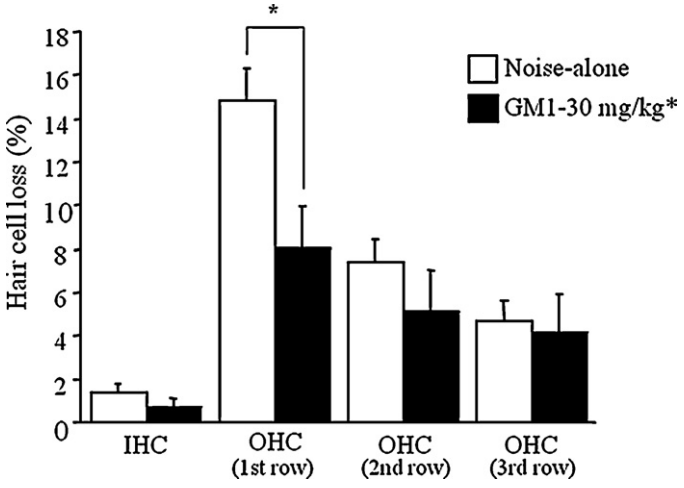

Fig. 3. The effect of 30 mg/kg GM-1 ganglioside on hair cell loss after acoustic overexposure. Missing hair cells at the 66% region from the apex were calculated 2 weeks after acoustic overexposure. Treatment with GM-1 significantly decreased loss of OHCs, especially in the first row (two-way ANOVA: $^*p < 0.05$, 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.