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The Free Radical Scavenger Edaravone Protects Hair Cells against Aminoglycoside Toxicity

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Abstract Reactive oxygen species are involved in inner ear hair cell death induced by aminoglycosides. Therefore, inhibition of free radicals can prevent hair cell death. The antioxidant, edaravone has been used clinically in Japan. In this study, we evaluated whether edaravone can protect hair cells against aminoglycoside-induced cytotoxicity in cultured mouse utricles. Utricles from CBA/N mice were cultured in medium containing neomycin alone (1 mM), or neomycin plus edaravone. Hair cells cultured with neomycin alone were damaged after 24 hours. Survival of hair cells cultured with neomycin plus edaravone (30 or 100 μM) was significantly greater than that of hair cells cultured with neomycin alone. Immunohistochemistry showed the production of 4-hydroxy-2-nonenal in utricles cultured with neomycin but not in utricles cultured with neomycin plus edaravone (100 μM). These results suggest that the inhibition of reactive oxygen species production by edaravone protects hair cells. In addition, high concentrations of edaravone did no show cytotoxicity.

Key words: hair cell, aminoglycoside, hydroxy radical, Edaravone, ototoxicity

Introduction

Sensory hair cells are easily damaged by factors such as aminoglycosides, infection, and ischemia.[1] Mammalian hair cells do not regenerate resulting in lifelong auditory and vestibular dysfunction. Therefore, it is important to prevent the loss of hair cells to adequately treat patients with inner ear diseases.

Previous studies have indicated that hair cell death is related to oxidative stress. In addition, aminoglycosides are well-known ototoxic reagents. Ototoxicity induced by aminoglycosides is associated with the generation of free radicals.[2] Recently, a unique free radical scavenger, edaravone, was developed[3] and has been used in the treatment of cerebral infarction in Japan.[4] We reported that edaravone protects the inner ear against noise-induced trauma in animal models.[5][6] In the present study, we investigated the effect of edaravone on aminoglycoside-induced hair cell death with the utricles culture of mice. In addition, we studied the cytotoxicity of edaravone to hair cells.

Materials and methods

Animal use and care

Four to six-week-old male CBA/N mice with normal Prayer reflexes were obtained from Kyushu Animal Company (Japan). The experimental protocol was reviewed by the Committee for Ethics on Animal Experiments of the Yamaguchi University School of Medicine. Experiments were carried out in accordance with the guidelines of this committee and Japanese Federal Law No. #105 and Notification No. #6 of the Japanese government.

Organ culture of utricles and induction of hair cell death

Animals were deeply anesthetized with pentobarbital and immediately decapitated. The temporal bones were quickly removed, and the vestibular organs were dissected in medium, consisting of Eagle's basal medium (Invitrogen Corp., Carlsbad, CA) supplemented with Earle's balanced salt solution (Invitrogen Corp.) (2:1, v/v). Only utricles were placed into the culture medium, which consisted of Eagle's basal medium supplemented with Earle's balanced salt solution (2:1, v/v) and 5% fetal bovine serum (Invitrogen Corp.). The utricles were incubated free-floating in 24-well tissue culture plates for 12 or 24 hours at 37℃ in a 5% CO_2 and 95% air environment. To induce hair cell death, neomycin solution (10 mg/ml; Sigma-Aldrich Co., St. Louis, MO) was added to the culture wells, at a final concentration of 1.0 mM. To evaluate the effect of edaravone, the reagent (10-100 μM) was added into the medium 2 hours before the addition of neomycin. After the experiments, utricles were fixed in 4% paraformaldehyde (PFA) for 1 hour at room temperature (RT). Otoconia were gently removed from fixed utricles with a stream of phosphate-buffered saline (PBS) applied via 28-G needle and syringe. After a rinse in PBS, samples were used in the following assays.

Immunohistochemistry to label hair cells

Fixed utricles were then incubated in blocking solution (1% bovine serum albumin, 0.4% normal goat serum, 0.4% normal horse serum, 0.4% Triton X-100 in PBS) for 3 hours at RT. To label hair cells, a mouse monoclonal antibody against calmodulin (Sigma-Aldrich Co.) and a rabbit polyclonal antibody against calbindin (Chemicon, International, Inc. Temecula, CA) were used to label whole-mount samples. Samples were incubated overnight at 4℃ in primary antibody solution (calmodulin 1:150, calbindin 1:250 in blocking solution). After a wash in blocking solution, samples were incubated with secondary antibodies diluted in blocking solution as follows: Alexa 488-conjugated goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR) and Alexa-594 conjugated goat anti-rabbit IgG (1:500; Molecular Probes). After a rinse in blocking solution, utricles were mounted in Vectashield® (Vector Laboratories, Inc.) and coversliped.

4-hydoroxy-2-nonenal immunohistochemistry

To evaluate the production of reactive oxygen species, 4-hydroxy-2-nonenal (4-HNE) production was investigated. Twelve cultured utricles were divided into 3 groups. Four utricles were cultured in normal medium for 14 hours. Eight utricles were cultured in normal medium for 2 hours, and then cultured for 12 hours in the presence of neomycin (1 mM) or neomycin plus edaravone (100 μM) in the medium. Utricles were fixed in 4% PFA and incubated in a 1:100 dilution of anti-4-HNE mouse monoclonal antibody (Oxis International, Inc., Portland, OR) overnight at (4℃). After a rinse in blocking solution, the samples were incubated with Alexa-488 conjugated goat anti-mouse IgG and Texas Red-conjugated phalloidin (1:100, Sigma-Aldrich Co) for 4 hours at RT. Samples were mounted in vectashield and coversliped.

Preparation of edaravone solution

Edaravone (Mitsubishi Pharma Corp., Osaka, Japan) was used as an antioxidant in this study. Edaravone is unstable when dissolved in water. Therefore, it was dissolved in medium just before cultures were initiated.

Evaluation of residual sensory hair cells

Utricles were examined on a fluorescence microscope (XF-EHD2, Nikon, Tokyo, Japan) to evaluate the survival rate of hair cells. Calbindin-positive and calmodulin-positive cells were counted as hair cells in the striolar region and the extrastriolar region respectively. Labeled hair cells were counted in random fields of 8 squares 20 μm^2 in each utricle. The striolar and extrastriolar hair cell counts were each averaged to give a single striolar and a single extrastriolar hair cell density for each utricle examined. At least six utricles were examined for each experimental condition.

Results

Effect of edaravone on hair cell survival

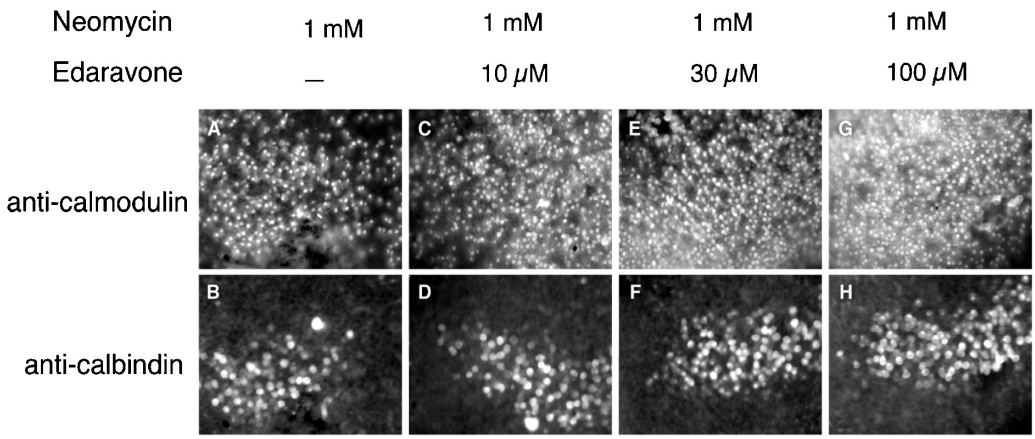

Fig. 1 Protective effect of edaravone on neomycin-induced cell death.
Utricles were cultured for 24 hours with neomycin (A, B) or with both neomycin and edaravone (C-H). Cell death of striolar hair cells (anti-calmodulin staining) and extrastriolar hair cells (anti-calbindin staining) was inhibited by edaravone.

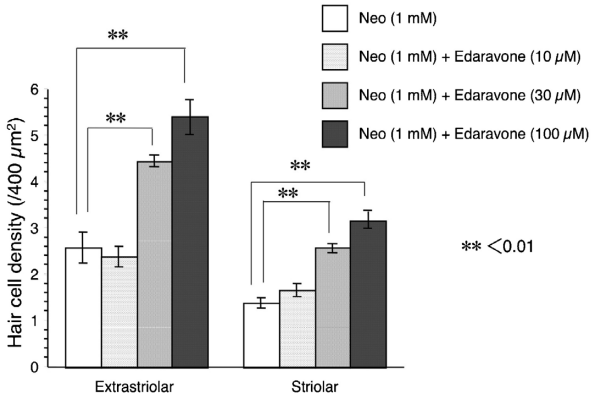

Fig. 2 Concentration-dependent effect of edaravone.
Hair cell density was significantly greater in utricles treated with 30 μM and 100 μM edaravone than in control utricles treated with neomycin alone. $^{*}p< 0.01$.

To evaluate the effect of edaravone on the survival of hair cells in response to neomycin treatment, utricles were cultured with neomycin (1 mM) alone or in the presence of edaravone (10-100 μM) for 24 hours. Utricles were fixed, and calmodulin and calbindin were immunolabeled to detect residual hair cells (Fig. 1). In response to neomycin alone, hair cells density was decreased. Hair cells density was greater in the medium containing neomycin and edaravone than in the medium containing neomycin alone. Hair cells density was quantified in Fig. 2. Edaravone inhibited the decrease of hair cells induced by neomycin. The effect of edaravone showed a dose-response relation; concentrations of 30 and 100 μM showed significant protection hair against neomycin cytotoxicity.

Edaravone inhibits the production of 4-HNE

To detect the production of hydroxy radical, immunohistochemistry was performed with an antibody against 4-HNE, a metabolic

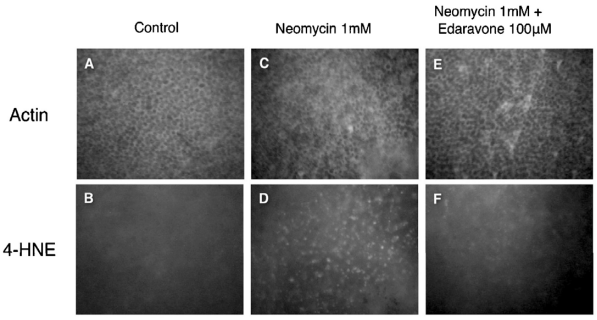

Fig. 3 Inhibition of 4-HNE production in hair cells by edaravone.
Utricles were cultured for 12 hours without neomycin (A, B), with neomycin (C, D), or with neomycin and edaravone (E, F). Hair cells layers were identified with Texas Red-conjugated phalloidin (A, C, E). 4-HNE was labeled with specific antibody (B, D, F). Immunostaining for 4-HNE was observed in utricle hair cells 12 hours after exposure to neomycin. This staining was inhibited by edaravone.

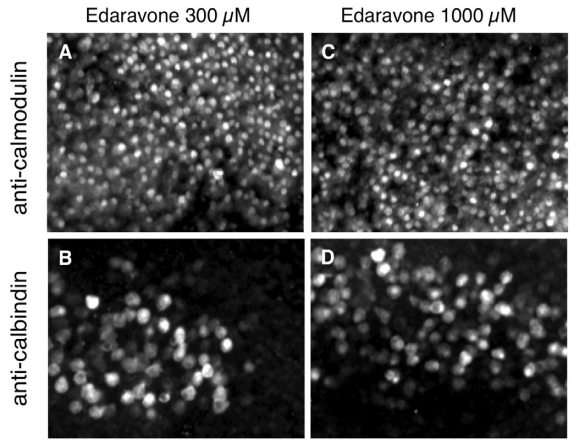

Fig. 4 High concentrations of edaravone have no cytotoxic effect.
High concentrations of edaravone showed no cytotoxicity in hair cells of cultured utricles.

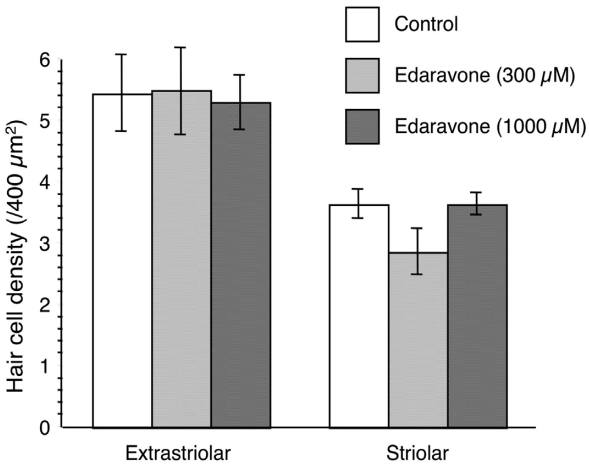

Fig. 5 Hair cell density is not affected by high concentrations of edaravone.
Utricles cultured with high concentrations of edaravone and control utricles showed similar hair cell densities.

product of hydroxy radical (Fig. 3). Utricles cultured for 12 hours were used in these experiments, because incubation in 1 mM neomycin for 24 hours decreased hair cell density. Beta-actin was labeled with phalloidin conjugated to Texas Red to indicate the hair cell layer. The 4-HNE staining was not observed in utricles cultured in the absence of neomycin (Fig. 3A, B). However, the 4-HNE staining of hair cells was observed in response to 1 mM neomycin (Fig. 3C, D). The 4-HNE staining was not observed in the utricles cultured with neomycin and edaravone 100μM for 12 hours (Fig. 3E, F). These results indicated that edaravone inhibited the production of hydroxy radical in response to neomycin.

Evaluation of high concentration of edaravone

To evaluate possible cytotoxic effects of edaravone, utricles were cultured for 24 hours with high concentrations of edaravone (300 μM, 1000 μM). The concentration of 1000 μM was maximum because edaravone could not be dissolved in the medium at higher concentrations. Hair cells were labeled with antibodies against calmodulin and calbindin. Hair cell densities were similar to those in control medium (Figs. 4 and 5).

Discussion

In this study, we showed that edaravone inhibits hair cell death induced by neomycin. Reactive oxygen species (ROS) play important roles in hair cell death induced by aminoglycosides.[7)8)] Aminoglycosides form the chelated metal complexes with iron ion after influx into hair cells. These complexes generate ROS such as hydroxy radical in hair cells. Threrefore, aminoglycosides are known to be specific ototoxic agents.[9)10)]

It was known that the activation of signal molecules related with apoptosis, after the generation of ROS in hair cells. The activation of JNK and caspase-9 were reported in hair cell death induced by aminoglycosides. These studies showed that the generation of ROS was upstream of the signaling system in hair cell death, and antioxidant is the candidates of reagents which can protect hair cells against aminoglycosides.[11)]

Many kinds of antioxidants have been tested for the ability to inhibit hair cell death induced by aminoglycosides, which can lead to hearing loss and vestibular dysfunction.[12)] Edaravone was the first antioxidant used in clinical practice in Japan. It was used to treat acute cerebral infarction.[4)] We reported that edaravone protects the inner ear against acoustic over stimulation in an animal model.[5)6)]

Peritoneal administration of edaravone prevents inner ear damage after intense noise exposure. The present study was designed to investigate the ototoxicity of edaravone and decide the optimum concentration of the reagent against inner ear tissue.

Watanabe[13] reported that edaravone possesses hydroxy radical scavenging activity and inhibits salicylate hydroxylation (IC_{50} = 6.7 μM). Edaravone (50 μM) also prevents lipid oxidation *in vitro*[4] and prevents cell death induced by 15-HPETE *in vitro*.[3] In the present study, the optimum concentration against neomycin-induced hair cell death was 30 - 100 μM.

We assessed the production of 4-HNE, which is a metabolite of the hydroxy radical. In hair cells exposed to aminoglycoside, strong 4-HNE staining was observed. Edaravone inhibited 4-HNE staining, suggesting that edaravone protects hair cells scavenging free radicals.

We also evaluated the possible cytotoxicity of edaravone and found none, up to 1000 μM. A clinical trial of edaravone for the treatment of inner ear diseases is under way in Japan.

Conclusion

In this study, we showed the optimum concentration of the reagent, edaravone against the vestibular hair cells. The reagent can prevent hair cell death induced by aminoglycoside. In addition, the reagent did not show the ototoxicity at the high concentration of 1000 μM.

Acknowledgements

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