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Topographic and quantitative evaluation of gentamicin-induced damage to peripheral innervation of mouse cochleae

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ABSTRACT

Ototoxicity induced by aminoglycoside antibiotics appears to occur both in hair cells (HCs) and the cochlear nerves that innervate them. Although HC loss can be easily quantified, neuronal lesions are difficult to quantify because two types of afferent dendrites and two types of efferent axons are tangled beneath the hair cells. In the present study, ototoxicity was induced by gentamicin in combination with the diuretic agent furosemide. Neuronal lesions were quantified in cochlear whole-mount preparations combined with microsections across the habenular perforate (HP) openings to achieve a clear picture of the topographic relationship between neuronal damage and HC loss. Multiple immunostaining methods were employed to differentiate the two types of afferent dendrites and two types of efferent axons. The results show that co-administration of gentamicin and furosemide resulted in a typical dynamic pattern of HC loss that spread from the basal turn to the outer hair cells to the apex and inner hair cells, depending on the dose and survival time after drug administration. Lesions of the innervation appeared to occur at two stages. At the early stage (2–4 days), the loss of labeling of the two types of afferent dendrites was more obvious than the loss of labeled efferent axons. At the late stage (2–4 weeks), the loss of labeled efferent axons was more rapid. In the high-dose gentamicin group, the loss of outer HCs was congruent with afferent dendrite loss at the early stage and efferent axon loss at the late stage. In the low-dose gentamicin group, the loss of labeling for cochlear innervation was more severe and widespread. Thus, we hypothesize that the gentamicin-induced damage to cochlear innervation occurs independently of hair cell loss.

1. Introduction

Ototoxicity, including cochleotoxicity and vestibulotoxicity, is one of the major side effects and limiting factors of the clinical use of aminoglycosides. However, some antibiotics in this category, such as gentamicin, are still widely used because of their efficacy in the treatment of some infections, such as tuberculosis and those caused by Gram-negative bacteria (Edson and Terrell, 1999; Sweileh, 2009). Gentamicin is also used to treatment Duchenne muscular dystrophy (Yukihara et al., 2011), and it is often co-administered with other drugs in seriously ill patients (Robertson et al., 2006).

Gentamicin selectively kills inner ear sensory hair cells (HCs) and neurons and causes subsequent sensorineural hearing loss. Although significant information concerning this toxicity exists, many issues regarding aminoglycoside ototoxicity in general, particularly in gentamicin-induced cochlear lesions, remain unclear. For example, most previous studies have focused on HC lesions rather than lesions of neuronal innervation (Stepanyan et al., 2011; Alharazneh et al., 2011; Ding et al., 2010). In the few

Abbreviations: HC, hair cell; OHC, outer hair cell; IHC, inner hair cell; HP, habenular perforate; SGNs, spiral ganglion neurons; MOC, medial olivary complex; LOC, lateral olivary complex; TMRD, tetramethylrhodamine-conjugated dextran; DAPI, 4′,6-diamidino-2-phenylindole; OSL, osseous spiral lamina; B, basal segment; M, middle segment; A, apex segment.

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studies that addressed neuronal lesions, the damage to spiral ganglion neurons (SGNs) induced by aminoglycoside has often been reported as secondary to the loss of HCs (McFadden et al., 2004; Takeno et al., 1998; Duan et al., 2000; Bae et al., 2008). However, several lines of evidence suggest that spiral ganglion neuron (SGN) damage might be independent of HC death. First, the extent and locations of SGN death are not always correlated with HC loss in animal studies of aminoglycoside ototoxicity (Sone et al., 1998; Hinojosa and Lerner, 1987; Ryals and Westbrook, 1988; Keithley et al., 1989; Bao et al., 2005; Kujawa and Liberman, 2009). In human subjects, one study showed that patients with a significant reduction in the number of SGNs had limited loss of HCs after long-term use of aminoglycoside antibiotics (Sone et al., 1998). In another report in humans, approximately one-third of SGNs were lost in two young adults after a short course of aminoglycoside therapy, whereas the HCs in the organs of Corti were essentially normal (Hinojosa and Lerner, 1987). Second, type I SGNs have been reported to die even without inner HC (IHC) loss in animals with acoustic trauma (Kujawa and Liberman, 2009). Finally, in a recent study, widespread IHC loss was established through dietary restriction of thiamine in the cochleae of mice lacking the high-affinity thiamine transporter; this restriction did not cause SGN death after several months, suggesting that SGN survival does not require IHCs (Zilberstein et al., 2012).

Gentamicin is known to cause damage to both HCs and neuronal innervation to the cochlea. However, no detailed information regarding the relationship between the damage to HCs and their neuronal innervation is available. This is due largely to the lack of reliable methods of quantifying the cochlear innervation, which consists of both afferent and efferent systems that are both further divided into two subtypes. The two types of afferent neurons (SGNs) are located in the Rosenthal canal of the cochlea, and the two groups of efferent neurons are located in the lower auditory brain stem. A single IHC is innervated by 15–25 type I SGNs, which comprise ~90–95% of the entire SGN population. By contrast, one type II SGN innervates 30–60 OHCs via en passant innervation. Efferent axons from medial olivary complex (MOC) neurons project to the OHCs and form axosomatic synapses; the efferent axons from lateral olive complex (LOC) neurons project to a region below the IHCs and form axodendritic synapses with type I SGNs at their terminals with IHCs (Rubel and Fritzsch, 2002). Type I SGNs are the principal encoders of the auditory signal. Type II SGN innervation is likely to provide sensory feedback from the OHC region as part of a neural control loop involving the efferent system (Darrow et al., 2007; Thiers et al., 2008; Glowatzki and Fuchs, 2000).

To enumerate these innervations and HCs, we microsectioned across the openings of the habenula perforate (HP) using a whole-mount preparation of the basilar membrane after counting HCs. This method ensures a clear topographic relationship between the locations of HC loss and damage to cochlear innervation. Moreover, the increased exposure of the openings of HP in the sectioned samples to the antibodies improves the immunostaining of the nerve fibers.

Gentamicin ototoxicity can be greatly enhanced by co-administration of loop diuretics such as ethacrynic acid and furosemide (Takeno et al., 1998; Duan et al., 2000; Mulheran and Harpur, 1998; Song et al., 2009). This synergistic interaction between gentamicin and loop diuretics is interesting because these two drugs are co-administered clinically and in research to establish gentamicin-cochlear lesions in animal models (Oesterle et al., 2008; Taylor et al., 2008). A single dose of gentamicin at 125 mg/kg (intramuscularly) in combination with ethacrynic acid at 40 mg/kg (intravenously) creates cochlear lesions that are comparable to those established by 600–900 mg/kg gentamicin alone (McFadden et al., 2004). Moreover, this combination approach significantly reduces mortality compared with the use of gentamicin alone to induce cochlear lesions (McFadden et al., 2004).

Cochlear lesions were established in the present study using the combination of gentamicin and furosemide to investigate the temporal relationship between HC loss and damage to cochlear innervation. Using the processing methods mentioned above in combination with staining for multiple factors, damage to cochlear innervation was documented quantitatively and might occur independently of HC damage.

2. Materials and methods

2.1. Animals and the drug-lesion paradigm

All procedures were approved by the Animal Ethics Committee of Shanghai Jiao Tong University, Shanghai, China. In total, 168 male and female C57/BL6 mice were recruited at 35 postnatal days after otological evaluations to exclude abnormalities of the outer and middle ears. Mice were grouped according to gentamicin treatment and the targeted observations as shown in Fig. 1. A total of 112 mice were divided equally into two groups according to the gentamicin dose (30 or 60 mg/kg). Gentamicin (Sigma–Aldrich, St. Louis, MO, USA) was administered subcutaneously in a single injection, and furosemide (400 mg/kg, intraperitoneally; Hospira Inc., Jiangsu, China) was administered 30–45 min later. The intoxication model was established in a pilot experiment. We used gentamicin doses of 30, 40, 50, 60, 80, and 100 mg/kg (10 male and female C57/BL6 mice per group). OHC and IHC loss increased with the gentamicin dose (Supplementary Figs. 1 and 2). When 80 mg/kg gentamicin was used together with furosemide, a rapid and consistent loss of OHCs resulted along virtually the entire length of the cochlea (Supplementary Fig. 2). When the gentamicin dose was increased to 100 mg/kg, more than a half of the animals were euthanized in 4 days. In the main experiment, the remaining 56 mice were used as age-matched controls and were administered vehicle (double-distilled water) injections. A total of 96 mice (32 from each of the three groups) were used to quantify the HC lesions and afferent and efferent peripheral innervation. Eight of the thirty-two mice from each group were euthanized sequentially at four time points (Fig. 1). The remaining 72 mice (24 from each of the three groups) were used to quantify SGNs, and eight mice from each group were euthanized at three sequential time points (Fig. 1).

The staining methods for the various neuronal structures are detailed in Table 1. All cochleae were first treated with tetramethylrhodamine-conjugated dextran (TMRD) tracer, which stains type I SGNs and all nerve fibers but not type II SGNs or their dendrites (Huang et al., 2007; Fritzsch, 1993). For the HCs, the whole-mount preparations of the basilar membranes were stained with 4′,6-diamidino-2-phenylindole (DAPI). After counting the HCs and MOC fiber crossings in the tunnel of Corti, the whole-mount preparations were sectioned across the HP openings. The sections were then stained with anti-peripherin antibody, which is specific for type II afferent dendrites, or anti-ChAT antibody, which is specific for efferent axons (Sobkowicz and Emmerling, 1989; Hafidi, 1998; Schimmang et al., 2000). The TMRD neuronal tracing in combination with immunochemical staining showed results consistent with but more detailed than the ultrastructural data (data not showed). To quantify the two types of SGN, the whole-mount preparations were treated with anti-peripherin antibody. The SGNs in the Rosenthal canals were counted under a confocal microscope as described below.