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Ephrin A2 protein expression in the regeneration and plasticity of cochlear hair cells in chicken following kanamycin ototoxicity★

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Abstract

The results from this study showed that the thresholds of brainstem auditory-evoked potentials peak following 10 successive days of intramuscular injection of Roman chickens with kanamycin, starting 3 days after birth. Fluorescence immunohistochemistry analysis revealed few ganglion cells positively labeled for Ephrin A2 in the cochlea of experimental chickens from 2 days before until 7 days after the last kanamycin injection. The number of Ephrin A2-positive ganglion cell bodies was increased at 15 days after the last injection and was similar to that in normal chickens at 30 days following the cessation of kanamycin treatment. These experimental findings indicate that Ephrin A2 protein expression in the acoustic ganglia is synchronized with the connection damage and regeneration of cochlear hair cells after kanamycin exposure. Ephrin A2 may play an important role in the regeneration and plasticity of cochlear hair cells in the chick cochlea following kanamycin ototoxicity.

Key Words: Ephrin A2; cochlear hair cells; reinnervation; plasticity; inner ear; neural regeneration

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INTRODUCTION

In contrast to the permanent nature of mammalian deafness, the avian auditory organ readily loses hair cells and becomes reinnervated after acoustic trauma[1] or ototoxic drug damage[2]. Moreover, in association with this regeneration and reinnervation, there is a recovery of hearing ability[3]. Ephrins, a well characterized family of membrane-bound axon guidance molecules[4], are involved in a diverse array of cell-cell interactions in the nervous system, establishment of topographic projections[5-8], and the repair of injured nerve tissue[7, 9-12]. However, the changes in Ephrin A2 protein expression in ganglion nerve cells of the avian cochlea after kanamycin damage, and especially during the reinnervation of regenerated hair cells following aminoglycoside ototoxicity, have not been investigated to date.

Therefore, this study sought to elucidate the changes in Ephrin A2 protein expression in the acoustic ganglion of the chicken cochlea following kanamycin ototoxicity and their correlation with the damage, regeneration and plasticity of regenerated hair cells undergoing reinnervation.

RESULTS

Quantitative analysis of experimental animals

A total of 66 newly hatched Roman chickens (aged 3 days; male or female) were equally, randomly divided into kanamycin and control groups. Chickens in the kanamycin group were intramuscularly injected with kanamycin sulphate at a dose of 200 mg/kg daily for 10 days. Control chickens received no injections. New animals were added if chickens died accidently during the experiment, to ensure that there were six chickens tested in each of the following groups: kanamycin group at 2 days before treatment and 1, 3, 7, 10, 15, 21, 30 days after kanamycin treatment; control group at 3, 13 and 43 days after birth.

Auditory brainstem response (ABR) measurements in chickens following kanamycin ototoxicity

In normal chickens, the ABR thresholds measured at 13 days were significantly lower than those measured at 3 days (71.6 ± 2.6 dB *vs*. 82.9 ± 4.4 dB, $P < 0.01$). Thresholds measured at 43 days were similar to those measured at 13 days after hatching (70.1 ± 3.3 dB, $P > 0.05$). This is evidence that the hearing function of

chickens was already mature by 13 days after hatching. In kanamycin-treated chickens, the ABR thresholds measurement showed that the hearing impairment occurred at the highest threshold after 10 days of kanamycin treatment (116.3 ± 4.3 dB). The hearing function of chickens began to recover following drug termination and was stable within 10 days after completion of the drug injection. There was a significant difference in ABR thresholds measured at 1 day (116.3 ± 4.6 dB) and 3 days (112.0 ± 5.2 dB) following termination of kanamycin treatment ($P < 0.05$). Thresholds measured at 7 days (101.5 ± 4.3 dB) were significantly lower than those measured at 3 days after drug cessation ($P < 0.01$), and those measured at 10 days (94.3 ± 4.8 dB) were lower than those measured at 7 days ($P < 0.01$). The ABR thresholds measured at 30 days were still higher than those in normal chickens (85.3 ± 2.8 dB *vs*. 70.1 ± 3.3 dB, $P < 0.05$).

Ephrin A2 expression in the acoustic ganglia of chickens following kanamycin ototoxicity

Immunohistochemical staining showed that nearly all ganglion cell bodies in normal control chickens were labeled positively for Ephrin A2 protein and the labeling was clearly nonhomogeneous (Figure 1). No significant differences in the numbers of Ephrin A2-positive cells were observed at different time points. The average number of immunopositive ganglion cells was about 160 in a 200-fold field (Figure 2).

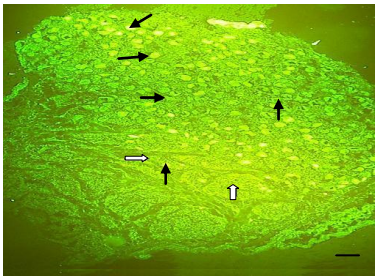

Figure 1 Ephrin A2 expression in frozen sections of chick acoustic ganglia from normal (control) animals at 13 days after hatching (immunohistochemistry). Nearly all ganglion cell bodies in control chicken were labeled positively for Ephrin A2.

Black arrows indicate strongly Ephrin A2-immunolabeled cells; white arrows indicate weakly Ephrin A2-immunolabeled cells. The fluorochrome is fluorescein isothiocyannate, the presence of which is detected as green fluorescence. Scale bar: 20 μm.

In the kanamycin group, there was a statistically significant difference in the number of Ephrin A2-labeled cells in the acoustic ganglion after kanamycin exposure at different time points (Figure 3). Few positive ganglion cell bodies labeled for Ephrin A2 were visible in the chicken cochlea from 2 days before treatment until 7 days after the last kanamycin injection, with an average of < 20 immunopositive ganglion cells in each 200-fold field (Figure 4A). The number of Ephrin A2-positive ganglion cells increased obviously at 15 days following the last kanamycin injection, with an average number of 70 cells in each 200-fold field. By 30 days after the cessation of kanamycin treatment, the number of Ephrin A2-positive ganglion cell bodies per 200-fold field was 130, similar to that in normal controls (Figure 4B).

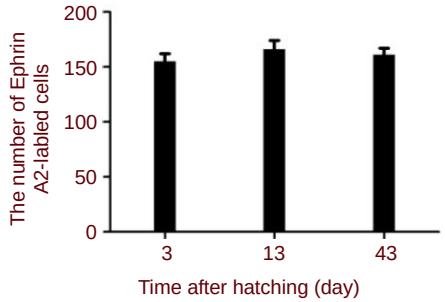

Figure 2 The average number of Ephrin A2-labeled cells (/200-fold visual field) in the acoustic ganglion from a normal (control) chicken at 3, 13 and 43 days after hatching under fluorescence microscope.

No significant difference was observed in the number of Ephrin A2-labeled cells at different time points. Data are expressed as mean ± SD and were analyzed with the Mann-Whitney *U* test.

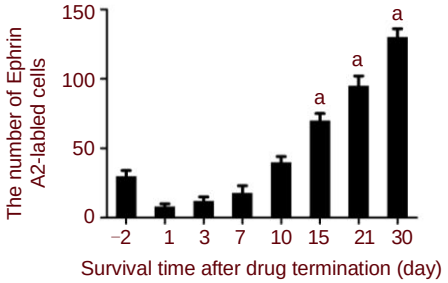

Figure 3 The average number of Ephrin A2-labeled cells per frozen section (/200-fold visual field) in the acoustic ganglia from kanamycin-treated chickens at 2 days before and 1, 3, 7, 10, 15, 21 and 30 days following termination of kanamycin treatment under fluorescence microscope.

−2 days: 2 days before the termination of treatment. Data are expressed as mean ± SD and were analyzed by the Mann-Whitney *U* test. $^aP < 0.05$, *vs*. kanamycin group at 2 days before and 1, 3 and 7 days after the termination of kanamycin treatment.

DISCUSSION

The morphology of regenerated hair cells in the chicken cochlea undergoing regeneration and reinnervation following kanamycin ototoxicity was described in our previous work. Scanning electron microscopy study showed that damage began at the proximal region of the cochlea with loss of hair cells and their innervation when chickens were exposed to drugs for 8 days. Degeneration of hair cells and their innervation was complete in 40% of basilar papillae after 10 days of kanamycin administration[3, 13]. One day following the last injection, a few erupted immature regenerated hair cells already possessed afferent and efferent terminals. Following innervation, these regenerated hair cells

became mature. By 15 days following the last injection, the innervation pattern of regenerated hair cells was similar to that in age-matched normal controls. At 30 days after kanamycin damage, the regenerated hair cells were completely mature.

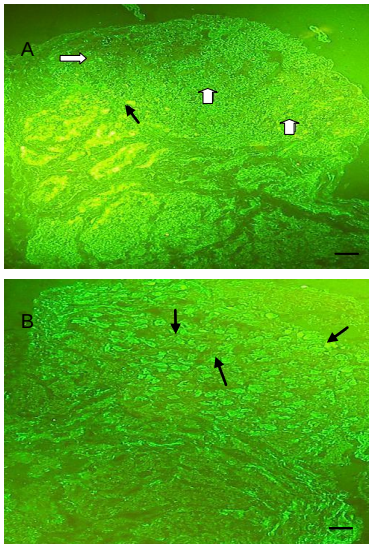

Figure 4 Ephrin A2 expression in frozen sections of the acoustic ganglia from a kanamycin-treated chicken at 1 day (A) and 30 days (B) following termination of kanamycin treatment.

The numbers of Ephrin A2-labeled ganglion cells (black arrows) decreased notably. White arrows show cells without Ephrin A2 expression. The fluorochrome is fluorescein isothiocyannate, the presence of which is detected as green fluorescence. Scale bars: 20 μm.

In this study, Ephrin A2 expression in the acoustic ganglia of chickens was determined at several time points after kanamycin injury and quantified by fluorescence immunohistochemistry. In chickens exposed to drugs for 8 days, because the damage occurred in the proximal region of the basilar papilla, the level of Ephrin A2 protein expression in the cochlea was lower than that in control chickens. After 10 days of kanamycin administration, when Ephrin A2 expression fell to the lowest level, complete degeneration of hair cells and their innervation was observed. This resembled Lee and Warchol's finding[12] that Ephrin A2 expression was lost from a subset of ganglion cells after treatment with gentamicin. In addition, fluorescence immunohistochemistry demonstrated that the number of Ephrin A2-positive cells began to increase as soon as the innervation of regenerated hair cells in the basilar papilla began, which occurred gradually following the last injection. Furthermore, there was a significant increase in the number of Ephrin A2-labeled cells at 15 days after kanamycin treatment, coincident with the development of neuroplasticity of regenerated hair cells. By 30 days after kanamycin ototoxicity, Ephrin A2 expression in the acoustic ganglion had reached a constantly normal level, accompanied by the maturation of innervation of the regenerated hair cells. These results show that the Ephrin A2 expression at several time points after kanamycin injury was synchronized with sensory regeneration and reinnervation, indicating that Ephrin A2 may play an important role in the regeneration and plasticity of reinnervation of the regenerated hair cells in the chicken cochlea following kanamycin ototoxicity.

In our previous studies on morphology, neither efferent nor afferent terminals could be seen on these unerupted hair cells after kanamycin damage[3]. Because nerve terminals are mainly composed of axolemma and flowing axioplasm, it is difficult to determine if several terminals are being observed at the same time. However, both afferent and efferent terminals on the hair cells appeared to stem from the acoustic ganglion. Therefore, it was worthwhile to investigate the relationship between the changes in the levels of some factors expressed in the acoustic ganglia and the related morphology. Ephrin and Eph receptors had been extensively implicated in various aspects of development of multiple organ systems[14-17], and their best known function is in the control of axonal guidance, neural circuit formation, topography, and plasticity[8, 18-19]. Several studies have addressed the involvement of different Ephrins and their Eph receptors in the formation of pathways in the avian ear and auditory brainstem, and the results of these studies have suggested a role for these molecules in the development and maintenance of appropriate innervation of cochlear hair cells[9, 20-21]. In addition, research on the relation between Ephrin A2 and the avian auditory nerve system demonstrated that Ephrin A2 is expressed throughout the eighth cranial nerve of the chick embryo during the growth of axons to the brainstem, and into the region of the auditory nuclei[12]. In summary, the synchronization of Ephrin A2 expression in the acoustic ganglia with sensory regeneration and reinnervation suggested an important role for Ephrin A2 in the regeneration and remodeling of regenerated hair cells in the chick cochlea following kanamycin ototoxicity. Based on the results of Hansen's study[22], Ephrin A2 inhibits retinal axonal growth at high concentrations but promotes growth at lower concentrations; therefore, we assumed that the decrease in Ephrin A2 expression in the chicken cochlea following kanamycin damage promotes innervation of regenerated hair cells and that increased Ephrin A2 expression accelerates the loss of surplus synapses at regenerated hair cells. It is therefore possible that Ephrin A2 expression underlies the plasticity of regenerated hair cells undergoing reinnervation. This hypothesis needs to be further studied.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

This experiment was performed at the Department of Otlaryngology & Head and Neck Surgery of Xinhua Hospital, Ear Institute, Shanghai Jiao Tong University

School of Medicine, China, from May 2009 to May 2010.

Materials

A total of 66 newly hatched Roman chickens, aged 3 days, of either gender, were housed in an animal laboratory of ordinary grade, Shanghai Guixing Breeding Chick Farm, China, and allowed free access to food and water. The animal protocol complied with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China in 2006[23].

Methods

Establishment of kanamycin ototoxicity models

Chickens in the kanamycin group were injected with kanamycin sulphate (Sigma, St. Louis, MO, USA) at a dose of 200 mg/kg daily[3] for 10 days. Injections were subcutaneously given once a day starting 3 days after birth.

Auditory testing

At the end of the survival period, ABR measurements were performed in kanamycin group animals at 2 days before and 1, 3, 7, 10, 15, 21 and 30 days following the last injection, and in control animals at 3, 13 and 43 days after hatching. All of the recordings were conducted in anesthetized chickens (administered *via* intraperitoneal injection of 10% chloral hydrate at a dose of 200 mg/kg) placed in a sound-proof chamber. Before recording sessions, chickens were examined for signs of middle ear infection, middle ear effusion, and accumulation of debris in the external auditory canal. The ABRs were measured using a Keypoint physiological response recorder (Alpine BioMed ApS, Vaidya, Denmark). Responses to 200 stimulus presentations were amplified, filtered (50–3 000 Hz band pass) and averaged. Using the Keypoint recorder, the analysis time was 10 ms and sensitivity was 10 μV/d. Sterile stainless steel pin electrodes were inserted into the bilateral mastoid subcutis as a reference and ground electrodes were placed in the contralateral ear, with the middle point of the cranium top as an active electrode. A half-cycle sine wave (4 000 Hz, produced by an SMP-3100 sound stimulation applicator; Nihon Kohden) delivered at a rate of 10 times per second through earphone placed 5 cm above the chicken head was used to evoke ABRs[24].

Expression of Ephrin A2 in acoustic ganglion as detected by immunohistochemistry

To detect the expression of Ephrin A2 in acoustic ganglia, immediately following hearing testing, chickens were decapitated and each cochlea was perfused with a 4% formalin/0.1 M phosphate solution *via* the round window of the cochlea. Acoustic ganglia prepared for frozen sections were processed as usual. Tissue specimens were cut into serial sections at a thickness of 20 μm at −20°C using a cryostat microtome (Leica CM1850; Leica Microsystems, Solms, Germany), placed onto microscope slides, and left to air-dry at room temperature for at least 15 minutes. The sections were incubated in blocking solution (2% normal goat serum, 1% bovine serum albumin, 0.1% Triton X-100 in phosphate buffer saline solution, pH 7.4) for 20 minutes at room temperature (−20°C). Subsequently, the sections were exposed to goat anti-Ephrin A2 polyclonal antibody (1:100; Dragonfly Sciences Co., Ltd., Shanghai, China) at 4°C overnight. After phosphate buffer saline rinses, the sections were incubated with fluorescein isothiocyannate-donkey anti-goat IgG (1:200; Dragonfly Sciences Co., Ltd., Shanghai, China) for 2 hours at room temperature in the dark, and cover-slipped for viewing. Specimens were examined by fluorescence microscopy (200 × magnification; Leica, Solms, Germany). Specimens were obtained from three frozen sections randomly selected from the acoustic ganglia to calculate the numbers of cells in all fields of vision on frozen sections, and the average value from three sections (200 × magnification) were considered representative.

Statistical analysis

Data are expressed as mean ± SD. The statistical significance of the changes in the average number of cells from three frozen sections showing Ephrin A2 reactivity was determined by analysis with the Mann-Whitney *U* test using SPSS 13.0 software (SPSS, Chicago, IL, USA). Two-tailed *P*-values of < 0.05 were considered to be statistically significant.

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Authors contributions: Jia Yu performed most of the experiments, most of the analysis and interpretation, and drafted the manuscript. Mingliang Xiang performed part of the study, coordinated the experiments, and contributed to the analysis and interpretation of the data. Hao Wu and Chenling Shen assisted in the study. All authors read and approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: This pilot study was approved by the Animals Ethics Committee of Xinhua Hospital, Shanghai Jiao Tong University School of Medicine in China.

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