

Protective effect of adenovirus-mediated erythropoietin expression on the spiral ganglion neurons in the rat inner ear

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Abstract. The aim of the present study was to evaluate the expression of erythropoietin (Epo) and the Epo receptor (Epo-R) in the spiral ganglion neurons (SGNs) of the rat inner ear, and to assess the effect of Epo adenovirus vector (Ad-Epo) on the spontaneous apoptosis of SGNs. A total of 60 ears from 30 healthy neonatal (2-3 days postnatal) Sprague-Dawley rats were used to examine the expression of Epo in the SGNs. The rats were divided into three groups: The negative control group, the vector control group [infected with a green fluorescent protein expression vector (Ad-GFP)] and the Ad-Epo group (infected with Ad-Epo). The expression of Epo and Epo-R was detected by immunohistochemistry and dual immunofluorescence staining using polyclonal antibodies directed against Epo and Epo-R, followed by confocal laser-scanning microscopy. An adenovirus vector was constructed and used to transfect the cultured SGNs. Following adenovirus infection, apoptosis of the SGNs was evaluated and Epo protein expression was assessed. Epo and Epo-R were widely expressed in the plasma membrane and the cytoplasm of the SGNs, as well as in the organ of Corti and the stria vascularis within the inner ear. Epo protein expression was upregulated in the Ad-Epo group compared with that in the other two groups ($P < 0.05$). Apoptotic cells were seldom observed at day 4 of SGN culture in the negative control group. At day 7, marked apoptotic cells were detected in the negative control group and the vector control group. The apoptosis level in the Ad-Epo group was significantly decreased compared with that in the negative control group or the vector control group at day 7 ($P < 0.05$). In conclusion, Epo and Epo-R are expressed in the SGNs of the inner ear of

the rat, and Ad-Epo can decrease the spontaneous apoptosis of SGNs, which may provide a basis for the prevention or alleviation of sensorineural hearing loss.

Introduction

Erythropoietin (Epo), a 34-kDa glycoprotein hormone, was originally characterized as the principal regulator of hematopoiesis due to its ability to inhibit apoptosis and stimulate the proliferation and differentiation of erythroid precursor cells (1,2). Epo was recognized as an engine for blood generation in various hematopoietic and non-hematopoietic mammalian tissues, including the central and peripheral nerve systems. Various experimental studies have shown that Epo exerts a marked neuroprotective effect *in vivo* and *in vitro* in nervous system disorders of the brain (3,4), spinal cord (5,6) and retina (7-9), among others.

Epo is initially produced in the liver and translocated to the kidney (10,11). Epo circulates in the blood stream and binds to its obligatory receptor, the Epo receptor (Epo-R), which is expressed in progenitor hematopoietic and non-hematopoietic cells, including endothelial cells, skeletal muscle cells and neural cells (12-14). Epo can exert an effect only by binding to the Epo-R homodimer, which alters the conformation of Epo-R, activates the phosphorylation of tyrosine-protein kinase JAK2 and Epo-R, and finally results in signal transduction involving signal transducer and activator of transcription 5A, phosphoinositide 3-kinase, mitogen-activated protein kinase and other signaling molecules (15). In the meantime, the binding of Epo and Epo-R may have an indirect influence on neuronal survival by modulating the pro-inflammatory environment and decreasing the subsequent neural apoptosis (5,16). Such findings from brain and spinal cord studies indicate that Epo and Epo-R are essential for neuroprotection. In addition, a previous study revealed that recombinant Epo most likely has otoprotective effects in newborn hypoxic-ischemic encephalopathy-induced cellular pathology and that it attenuates hearing loss (17).

Sound stimulation of the auditory hair cells (HCs) is transduced into electrical signals that are transmitted to the brain via the spiral ganglion neurons (SGNs). The cochlea in the inner ear transduces complex sound waves into electrical neural activity in the auditory nerve. The SGNs, the inner and outer HCs, and the stria vascularis (SV) are crucial elementary structures of the cochlea in the inner ear (18). Depolarization of the SGNs is initiated by the inner HCs once sound stimulation

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Abbreviations: Epo, erythropoietin; Epo-R, erythropoietin receptor; SGNs, spiral ganglion neurons; Ad-Epo, erythropoietin adenovirus vector; SNHL, sensorineural hearing loss

Key words: erythropoietin, erythropoietin receptor, spiral ganglion neurons, inner ear, adenovirus

has activated the complicated mechanism of auditory transduction (19). Degeneration and apoptosis of the SGNs leads to sensorineural hearing loss (SNHL) after deafening, which is associated with damage to the inner ear. Certain exogenous neurotrophins or neuroprotectants have been shown to be effective in preventing the degeneration or death of SGNs following SNHL (20,21). Certain studies have shown that using recombinant adenovirus vector-mediated gene therapy to provide certain neuroprotectants can enhance the survival rate or the regenerative sprouting of SGNs (22,23).

The potential neuroprotective effect of Epo in the inner ear depends on whether Epo and Epo-R are expressed in SGNs from the inner ear. However, the exact expression patterns of Epo and Epo-R in the rat inner ear and their role in hearing in general remain poorly understood. Additionally, there is a requirement for an effective interventional approach for enhancing Epo expression in the SGNs. The present study was designed to investigate whether Epo and Epo-R are expressed in the SGNs of the rat inner ear. An Epo adenovirus vector (Ad-Epo) was constructed to investigate the potential neuroprotective effect of Epo on the SGNs in the inner ear, with the aim of providing a basis for the prevention or alleviation of SNHL.

Materials and methods

Dissociation and preparation of cochlear specimens. A total of 30 healthy neonatal (2-3 days postnatal) Sprague-Dawley rats of a clean grade (weighing 6-8 g) were provided by the Animal Medical Center of the Third Military Medical University (Chongqing, China). Rats were maintained under standard laboratory conditions, at 18-27°C and 40-70% humidity under a 12-h light/dark cycle, with free access to clean water and food. The experimental protocol was approved and supervised by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (production license no. SCXK-PLA-20070015; occupancy permit no. SYXK-PLA-20070035). In this study, the guidelines for ensuring the welfare of laboratory animals were followed, and the welfare of the rodents was optimized by improving their living environments. The animals were maintained under standard laboratory conditions and were provided with clean water and food. To relieve their pain and fear, all rats were sacrificed by rapid decapitation.

Tissue preparation for immunohistochemistry. Dissection of the cochleae was performed as previously described (24,25). In total, 60 ears from 30 rats were used: 5 were sectioned for staining, 15 were used for the culture and the other 10 were used for the apoptosis test. Both ears from each rat were used.

The temporal bones were dissected to remove the cochleae, and the capsules were fractured to reveal the membranous cochlear duct and the bony modiolus. The modiolus with the encased acoustic ganglion neurons and the attached cochlear duct was dissected and transferred to a different Petri dish, where it was opened in 37°C Dulbecco's phosphate-buffered saline (PBS). The middle ear was then opened, and the cochlea was cautiously perfused with 1.5% paraformaldehyde. The fixative was slowly injected into the tympanic scale of the basal turn through the round-window plasma membrane. A small opening was made at the cochlear apex to allow cochlear perfusion. The specimens

were submersed in the same fixative at 4°C for at least 1 week to ensure complete fixation of the osseous tissue components. The cochlea was split into two by a longitudinal mid-modiolar trans-section and was subsequently embedded in paraffin. To optimize the immunostaining procedure, the concentration of the primary antibody was titrated, and different antigen-retrieval procedures, including microwave oven treatment using different buffers, and secondary visualization methods were investigated. Animals were anaesthetized with 100 mg/kg sodium pentobarbital and then perfused with 4% paraformaldehyde. The brain was dissected, removed and post-fixed overnight in the same fixative, and then transferred to 30% sucrose at 4°C until sinking to the bottom. The brain was sectioned in a coronal plane (5-10 μ m) on a paraffin machine. The brain tissues staining method is the same as that of inner ear.

Sections of 5-10 μ m in thickness were cut from the embedded tissue and dried at 60°C to melt the paraffin. De-paraffinization of the tissues was performed using xylene, and rehydration was achieved using decreasing concentrations of ethanol to water. All blocking and incubations were at room temperature. A 10-min Tris-buffered saline rinse was performed, and the endogenous peroxidase activity was blocked by incubation in 3% H₂O₂ for 5 min followed by a 30-min incubation with the primary antibodies: Goat anti-Epo antibody (N-19; 1:100; cat. no. sc-1310; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and rabbit anti-Epo-R antibody (1:50; cat. no. bs-1424R; Bioss, Inc., Woburn, MA, USA). Sections were subsequently incubated with anti-goat (1:150; cat. no. SP9000) or anti-rabbit (1:150; cat. no. SP9001) secondary antibodies from immunohistochemistry staining kits purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). The tissue was rinsed in running tap water for 5 min. Negative control staining was performed using hematoxylin and eosin (H&E) for 45 sec, followed by repeated rinses using running tap water.

Culture of SGNs. The majority of the protocols followed previously described methods (25). The cochleae were removed from the capsules, and the spiral ligament and SV were removed carefully. The spiral ganglion and modiolus were separated from the other peripheral tissue by a cut at the border between the spiral ganglion and the limbus. Once the spiral ganglia had been isolated, enzymatic dissociation was performed for 20 min in Hanks' balanced salt solution containing 0.1% trypsin and 0.01% DNase I (Roche Diagnostics GmbH, Mannheim, Germany) and was stopped using fetal calf serum (FCS) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The spiral ganglia were washed using culture medium prior to being mechanically dissociated, and this process was followed by trituration, as previously described (26). The culture medium was Dulbecco's modified Eagle's medium supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (both Invitrogen; Thermo Fisher Scientific, Inc.), glucose, penicillin and other components, including Hanks' solution (Hyclone, Logan, UT, USA), trypsin and collagenase I (both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and FCS (Invitrogen; Thermo Fisher Scientific, Inc.). The SGNs were seeded in the laminin-coated wells of a 96-well culture plate at 1x10⁵/ml.

Immunohistochemical analysis. The anti-neurofilament antibody (1:50; cat. no. ab129349) was obtained from Abcam

(Cambridge, UK). The primary antibodies, goat anti-Epo and rabbit anti-Epo-R were the same as those mentioned in the tissue preparation section. The dissected cochlea specimens were rinsed using PBS prior to incubation with a secondary antibody at a 1:2,000 dilution in 1.5% normal horse serum for 30 min at room temperature. The secondary antibodies were the same as those mentioned in the tissue preparation section. All the specimens were immunostained with an anti-neurofilament antibody to aid the visualization and identification of the SGNs and their processes. For primary staining, the cultures were incubated with the neurofilament antibody for 1 h at 37°C. When the presence of SGNs was confirmed, the anti-Epo and the rabbit anti-Epo-R antibodies were used in the subsequent protocol. The cells were rinsed using PBS prior to incubation with a secondary biotinylated antibody at a 1:2,000 dilution in 1.5% normal horse serum for 30 min at room temperature. Subsequent to washing with PBS, the cells were treated with the ABC complex solution (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's protocols. The staining was visualized using diaminobenzidine for 5 min at room temperature under a BHF342 fluorescence microscope (Olympus Inc., Tokyo, Japan). Controls were performed by eliminating the respective antibodies from the protocol, and no deposition of immunoreactive products occurred.

Dual immunofluorescence staining. The sections were fixed for 45 min at room temperature using 4% paraformaldehyde in PBS (24). The aforementioned Epo and Epo-R antibodies were used, and the secondary antibody for fluorescence detection was a mouse monoclonal anti-red fluorescent protein-tagged antibody (1:50; cat.no.CW0254; CWBio, Beijing, China). The sections were stained at room temperature with DAPI (Sigma-Aldrich; Merck KGaA) as a nuclear counterstain. The specimens were then rinsed for 3-10 min using PBS. Next, the specimens were incubated overnight at 4°C with primary antibodies diluted in blocking solution followed by incubation with species-specific secondary antibodies conjugated to fluorescein isothiocyanate or phycoerythrin. The samples were examined using a Leica model TCS SP5 confocal laser-scanning microscope (Leica Biosystems, Wetzlar, Germany). LAS-AF-Lite software (version 2.4.1_6384; Flexera Software, Inc., Maidenhead, UK) was used for image capture and deconvolution analysis.

Construction and transfection of Ad-Epo. Ad-Epo was prepared as previously described (27-29). The virus was grown to high titers in cells of the E1-containing human kidney 293 cell line. The buffer was replaced with 10 mM Tris (pH 7.5) and 1 mM MgCl. The virus was sterilized by passage through a sterile 0.2- μ m filter and frozen until use. The cultured SGNs were transfected with the recombinant adenovirus Ad-Epo. The SGNs were then seeded into six-well plates at 1×10^5 /ml and cultured for 48 h at 37°C. Subsequently, the serum-free medium DMEM/F12 (Hyclone) was changed and SGNs were harvested at the indicated time-points (at 24 or 48 h after Ad-Epo transfection respectively). SGNs grown in serum-free medium formed the negative control group, neurons that were infected with the green fluorescent protein expression vector (Ad-GFP) formed the vector control group, and neurons infected with Ad-Epo formed the Ad-Epo group.

Apoptosis of SGNs. SGNs were cultured under different conditions, collected and fixed using cold 80% ethanol at 4°C overnight. The SGNs were then resuspended in PBS for 15 min at room temperature, 1.0 ml of propidium iodide (PI) containing RNase A (10 mg/ml) was added and the SGNs were incubated at 37°C for 30 min in the dark. The apoptosis rate was determined at days 4 and 7 in the negative control group, at day 7 in the vector control group and at day 7 in the Ad-Epo group. The apoptotic cells, which were stained with PI, were detected via flow cytometry using a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) at an excitation wavelength of 488 nm.

Western blotting. At 48 h post-Ad-Epo infection, total protein from the cultured SGNs was extracted using a Protein Extraction kit (P0027; Beyotime Biotech, Beijing, China) and its concentration was determined using a BCA Assay kit (P0010; Beyotime Biotech). Samples (35 μ g) were loaded into 3-8% SDS-PAGE. Immunolabeling of the polyvinylidene difluoride membranes was performed as previously described (30). The membrane was blocked with 5% non-fat milk-TBST overnight at 4°C. The membrane was then washed with TBST and incubated with antibody recognizing Epo as aforementioned overnight at 4°C, followed by 3 washes with TBST and incubation with peroxidase-conjugated goat anti-mouse IgG (1:1,000; Zhongshan Biotech, Beijing, China) or goat anti-rabbit IgG secondary antibodies (1:1,000, Zhongshan Biotech) for 1.5 h at room temperature. Monoclonal anti- β -actin antibody (1:1,000; AA128; Beyotime Biotech) was used for the reference protein. ECL Plus kit (Pierce Inc., Appleton, WI, USA) was used for the detection of blot visualization and analyzed by Image Pro Plus software 5.0 (Media Cybernetics, Rockville, USA). The expression of SGNs was also labeled by GFP and observed prior to and following Ad-Epo infection.

Statistical analysis. Data analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). The results of the statistical analysis are expressed as the mean \pm standard deviation. Apoptosis rate and Epo protein level data were analyzed using one-way analysis of variance and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Immunohistochemical detection of Epo and Epo-R expression in the inner ear. The positive control for Epo immunohistochemical staining was the brain (Fig. 1A), whereas the negative controls for Epo and Epo-R immunostaining were inner ear sections stained only with H&E (Fig. 1B). Epo labeling was observed in the majority of the neurons of the inner ear as coarse granulated staining on the plasma membrane, in the cytoplasm surrounding the nuclei, which were stained blue (Fig. 1C), in the organ of Corti, including the HCs, and in the SV (Fig. 1D). Epo-R labeling was observed as clear granulated staining in the plasma membrane and cytoplasm of SGNs in the inner ear (Fig. 1E), in the phalangeal cells, in the organ of Corti (Fig. 1E and F), in the HCs and in the SV (Fig. 1F).

Scanning confocal fluorescence microscopy. Following immunohistochemical detection of Epo, inner ear specimens were stained for Epo using immunofluorescence and were

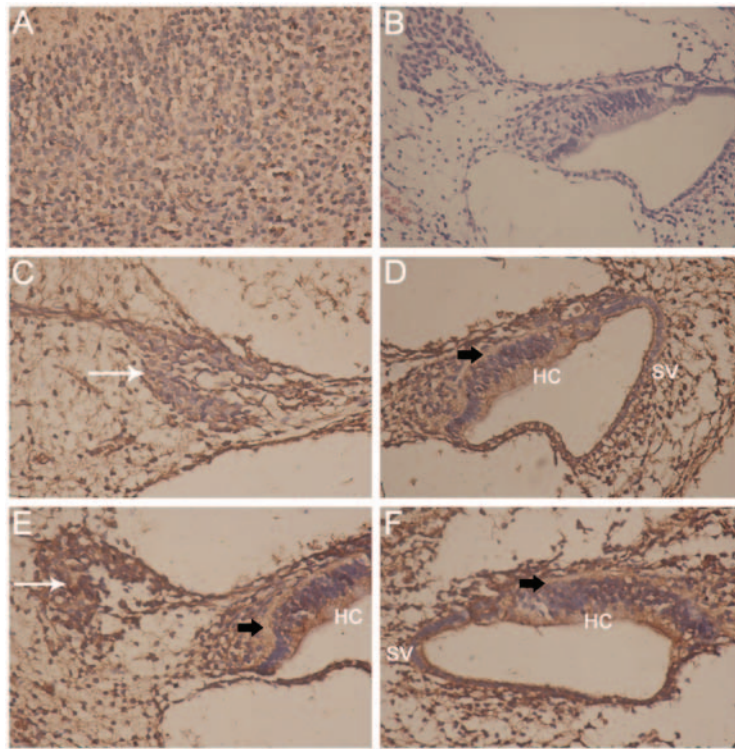


Figure 1. Micrographs showing Epo and Epo-R, and control staining. (A) Positive control staining of the brain (IHC; magnification, x400); (B) negative control staining of the inner ear (hematoxylin and eosin staining; magnification, x100); (C) Epo is localized in rat SGNs (white arrow; magnification, x400); (D) Epo is localized in the organ of Corti (black arrow), the HCs and the SV (magnification, x400); (E) Epo-R staining of SGNs (white arrow). One region of the organ of Corti (black arrow) is displayed, showing staining in the HCs (magnification, x400); (F) Epo-R staining of the entire organ of Corti (black arrow), showing Epo-R in the HCs and the SV (magnification, x400). Epo, erythropoietin; Epo-R, Epo receptor; IHC, immunohistochemical; SGNs, spiral ganglion neurons; HC, hair cell; SV, stria vascularis.

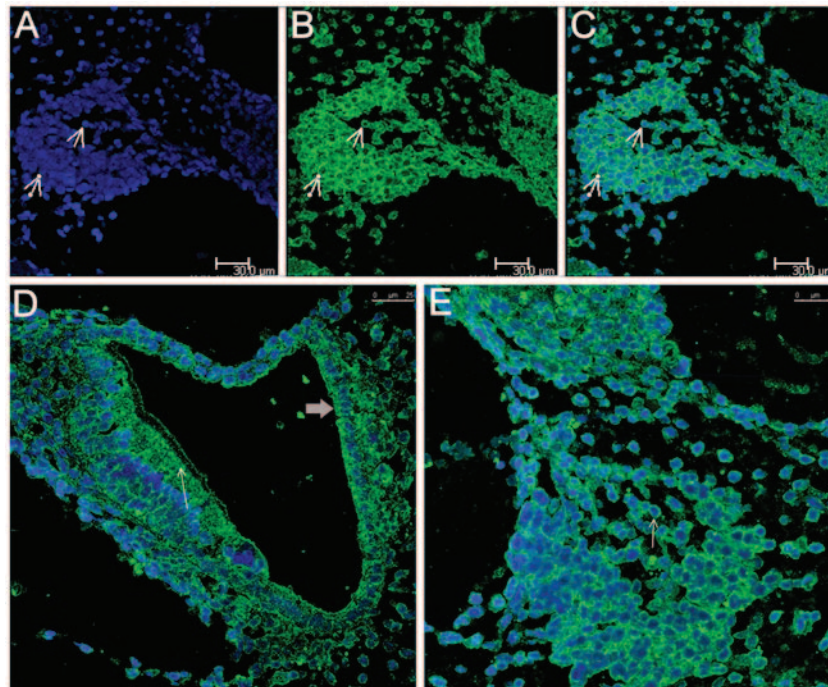


Figure 2. Immunofluorescence detection of Epo expression using confocal laser-scanning fluorescence microscopy. (A) The nuclei of the SGNs were stained with DAPI and appear blue (white arrow); (B) anti-Epo can be visualized in the membranes and in the cytoplasm surrounding the nucleus of the SGNs (green, white arrow); (C) merge of (A and B), showing that Epo is present in the SGNs (white arrow); (D) a merged image showing dual staining with DAPI and anti-Epo. Epo is expressed in the organ of Corti (long white arrow) and the stria vascularis (short gray arrow); (E) a magnified section of (C), displaying Epo in the SGNs (white arrow). Epo, erythropoietin; SGNs, spiral ganglion neurons.

observed under a confocal laser-scanning fluorescence microscope, which clearly revealed the blue DAPI-stained nuclei

of numerous SGNs (Fig. 2A). Positive labeling with anti-Epo appeared as fluorescence in the plasma membrane and in the

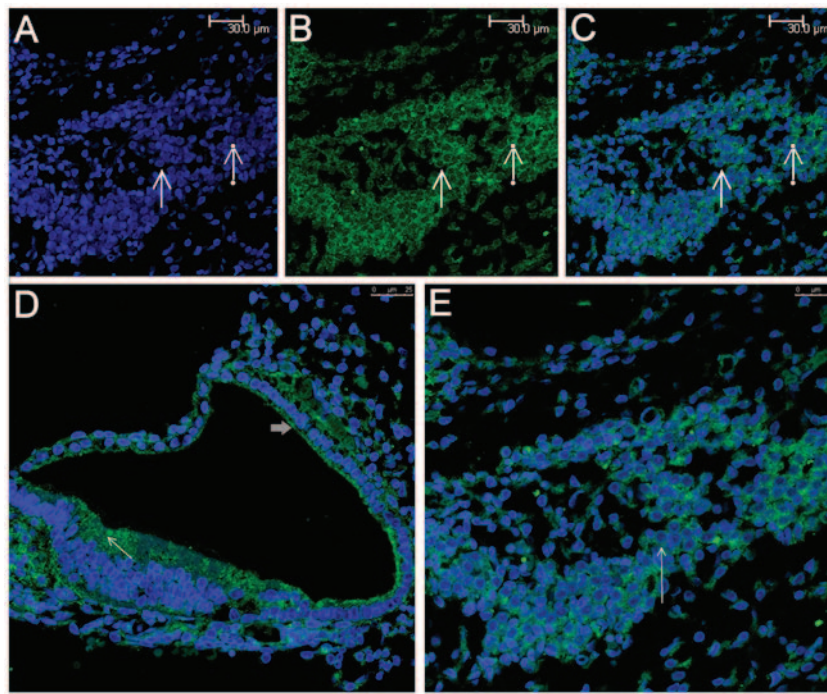


Figure 3. Immunofluorescence detection of Epo-R expression using confocal laser-scanning fluorescence microscopy. (A) The nuclei of SGNs were stained with DAPI and appear blue (white arrow); (B) anti-Epo-R can be visualized in the membranes and in the cytoplasm surrounding the nucleus of the SGNs (green, white arrow); (C) merge of (A and B), showing that Epo-R is present in certain SGNs (white arrow); (D) a merged image showing dual staining with DAPI and anti-Epo-R. Epo-R is expressed in the organ of Corti (long white arrow) and the stria vascularis (short gray arrow); (E) a magnified section of (C), displaying Epo in the SGNs (white arrow). Epo, erythropoietin; Ep-R, Epo receptor; SGNs, spiral ganglion neurons.

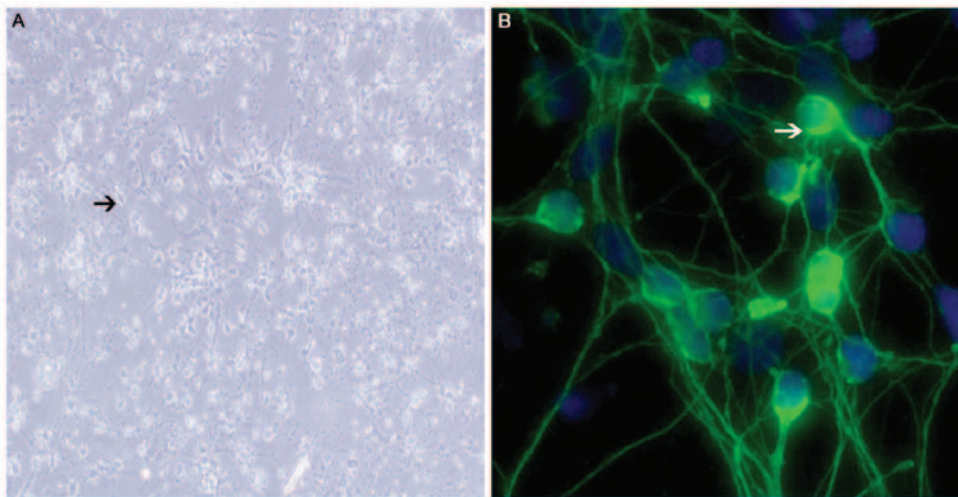


Figure 4. Micrographs of SGNs. (A) Bipolar neurons present in cultured SGNs (arrows; magnification, $\times 100$); (B) SGNs stained with anti-neurofilament protein, which is used for the identification of SGNs; green signals can be observed in the membrane (arrow; magnification $\times 200$) rather than in the nuclei. SGNs, spiral ganglion neurons.

cytoplasm surrounding the nucleus of the SGNs (Fig. 2B). The merged images display the localization of Epo in the SGNs in detail and clearly show that Epo was present on the plasma membrane and in the cytoplasm surrounding the nucleus of the SGNs (Fig. 2C). Additionally, Epo expression was also detected in the organ of Corti, including the phalangeal cells and the HCs, and in the SV (Fig. 2D). More details of Epo presentation in the SGNs can be seen in Fig. 2E.

Similarly, Epo-R was also detected in the SGNs (Fig. 3B and C), which was in agreement with the

results of a previous study (31), and in the organ of Corti (e.g., in HCs) and the SV (Fig. 3D). More details of Epo-R presentation in the SGNs can be seen in Fig. 3E. Based on a coarse comparison of the levels of Epo and Epo-R staining, it appeared that Epo was more highly expressed in the SGNs than Epo-R.

Identification of SGNs. SGNs were seeded into six-well plates and cultured. The cultured SGNs, which exhibited typical bipolar shapes (Fig. 4A and B), were stained with an

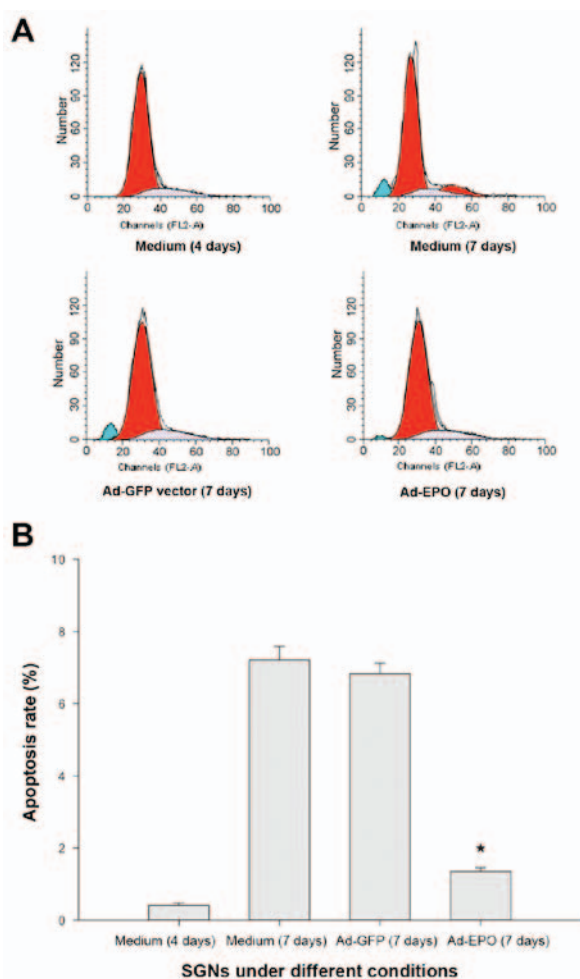


Figure 5. Effect of Ad-Epo on the apoptosis of the cultured SGNs. The cultured SGNs were collected and apoptosis was evaluated using flow cytometric analysis. (A) The rates of apoptosis (the shallow blue regions) were increased more significantly at day 7 in the serum-free medium in comparison with that at day 4, and remained unchanged after being infected by Ad-GFP (as a control), but showed a significant decrease after being infected by Ad-EPO at day 7. (B) The difference between the Ad-Epo group and the other groups was significant at day 7 ($P < 0.05$). Epo, erythropoietin; Ad-Epo, Epo adenovirus vector; Ad-GFP, green fluorescent protein expression vector; SGNs, spiral ganglion neurons.

anti-neurofilament protein antibody for identification. The cells displayed green fluorescence in the plasma membranes, but not the nuclei, confirming their identity as neurons (Fig. 4B).

Apoptosis of cultured SGNs. Detection of apoptosis among the SGNs was based on staining of the cells with PI, which can enter dead cells via permeable plasma membranes and intercalate into the DNA (Fig. 5A). Apoptotic cells were seldom observed at day 4 of culture of the SGNs in the negative control group. At day 7, marked apoptotic cells were detected in the negative control group and the vector control group. The rate of apoptosis observed in the Ad-Epo group was significantly lower than that observed in the negative control group or in the vector control group at day 7 ($P < 0.05$) (Fig. 5B).

Epo expression following Ad-Epo infection. The virus titer determined in the plaque assay using 293 cells

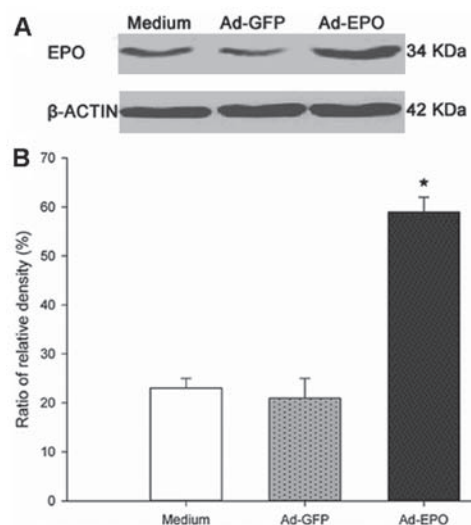


Figure 6. Effects of Ad-Epo on Epo protein expression in the cultured SGNs. SGNs in serum-free medium and SGNs infected with Ad-GFP were utilized as control groups. (A) Epo protein expression in neurons of the three groups; (B) transfection with Ad-Epo increased Epo protein expression in the cultured SGNs ($P < 0.05$). Epo, erythropoietin; Ad-Epo, Epo adenovirus vector; Ad-GFP, green fluorescent protein expression vector; SGNs, spiral ganglion neurons.

was 1.17×10^{11} IU/ml. The level of Epo protein in the SGNs was determined by western blotting 48 h after the SGNs were infected with the adenovirus. Epo protein expression was detected in the SGNs in the negative control group, the vector control group and the Ad-Epo group (Fig. 6A). Ad-Epo was capable of infecting SGNs, and Epo expression was upregulated in the SGNs in the Ad-Epo group compared with the levels in the other two groups ($P < 0.05$) (Fig. 6B). Expression of Epo labeled by GFP prior to and following Ad-Epo infection demonstrated increased Epo expression following infection. The staining in the micrographic views was lower in the non-infected SGNs (Fig. 7A), but higher in the SGNs infected by Ad-EPO (Fig. 7B).

Discussion

SNHL is a common and serious health problem (32,33) that largely results from various types of injuries to the HCs, SGNs or other structures of the inner ear (34,35). Serious and long-term SNHL is always incurable and irreversible; thus, potential methods for preventing damage to SGNs and other inner ear components, or possibly restoring their normal function, have attracted much attention (36-39). To the best of our knowledge, the present study is the first to examine the expression of Epo and Epo-R in the inner ear of neonatal rats and in isolated primary cultured SGNs, and to examine the effects of adenoviral transfection of isolated primary cultured SGNs.

Epo is the primary stimulator of red blood cell formation (11) and is a pleiotropic cytokine with neuroprotective effects that have been largely investigated in the central nervous system, particularly in the brain (40). Astrocytes have been shown to be responsible for the production of brain Epo (41), and Epo-R (42) is abundantly expressed in the brains of rodents (2,4,24,43,44). Epo is regarded as an important neuroprotective candidate for treating trauma, stroke, inflammation

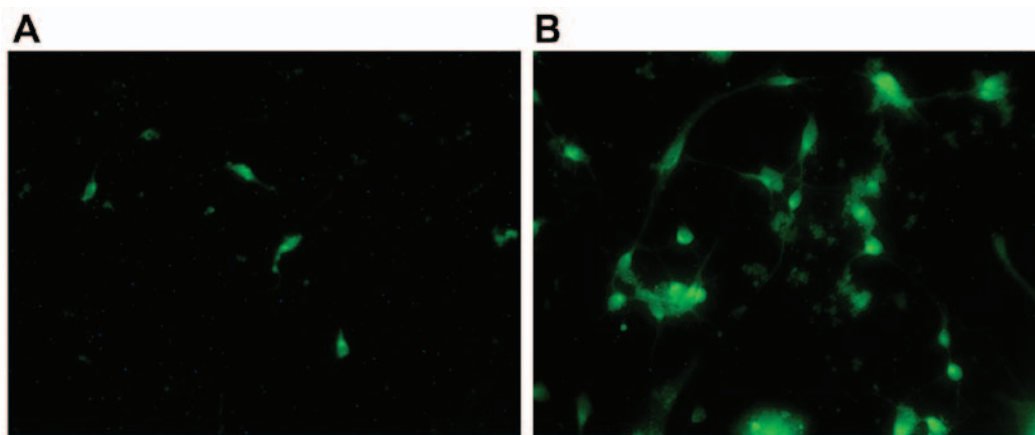


Figure 7. Expression of Epo was labeled by green fluorescent protein in spiral ganglion neurons (A) prior to and (B) following Epo adenovirus vector infection. Increased Epo expression can be observed (magnification, x200). Epo, erythropoietin.

and other impairments (3,16,26,45). The role of Epo and Epo-R in the SGNs and the inner ear remains unclear. It has been found that Epo and Epo-R are expressed in the inner ear of guinea pigs (24), and that Epo-R is expressed in the cultured rat SGNs (31). Other evidence for the potential protective effect of Epo is that it prevents amino glycoside-induced (46) and ischemia-induced HC death (47). The neuroprotective effect of Epo may involve at least two mechanisms, including a decrease in the HC death rate and an increase in the expression of angiogenic genes, such as vascular endothelial growth factor and C-X-C chemokine receptor type 4 (48). Epo has also been found to protect against gentamicin-induced auditory HC damage *in vitro* (49). Previous findings indicated that brain-derived neurotrophic factor and Epo may promote neuronal survival in the inner ear *in vitro* (50). Additionally, Epo was found to act as an otoprotectant in a DFNB12 mouse model with progressive hearing loss (51).

In the present study, it was demonstrated that Epo and Epo-R are expressed in SGNs and in other structures of the inner ear of normal neonatal rats. The observed wide distribution of Epo and Epo-R in the SGNs, the organ of Corti and the SV in the cytoplasm surrounding the nuclei is consistent with their localization in neurons (24,43). Considering the confirmed neuroprotective effect of Epo in the brain and its localization in the inner ear, it was concluded that Epo and Epo-R may be important neuroprotectants of SGNs in the inner ear.

However, contrary to our hypothesis, in a previous study, it was observed that Epo induced neurite outgrowth rather than increased survival of the SGNs. Epo was proposed as a possible candidate that could be used to enhance and modulate the regenerative effects of known neurotrophic factors on SGNs (31). However, Frederiksen *et al* (52) reported a result that contradicted the beneficial effects of Epo that had been reported by the vast majority of researchers. In this study, Epo was found to augment noise-induced hearing loss by altering the dynamics of blood flow to the cochlear vascular bed through potentially inducing vasoconstriction; the pathophysiological alterations also included reduced cochlear blood flow, such as localized periods of stasis, alterations in vascular permeability and local ischemia, which may result in temporary or even permanent deafness. The discrepancies between the results of this study and other earlier results may be associated with

the aforementioned unexpected finding from the present study that Epo and Epo-R are expressed in the SV. Further study is therefore required.

When examining the potential neuroprotective or cytoprotective roles of Epo and Epo-R in the inner ear, the blood-labyrinth barrier must inevitably be taken into consideration (36,46,53). The blood-perilymph barrier may preclude the passage of circulating Epo to the inner ear, although a local paracrine system may exist. Thus, the systemic administration of Epo, even at high doses, may not sufficiently increase the endocochlear concentration of Epo (24). *In vitro* investigations are therefore necessary. Cultures of dissociated SGNs may provide an experimental environment in which the molecular mechanism underlying the effects of Epo on SGNs may be examined. Differences in the culture preparations and conditions used in different studies are likely to underlie differences in the results. For example, the majority of neurons in adult spiral ganglia *in vivo* are bipolar. However, spiral ganglion cultures often exhibit a mix of neuronal morphologies, including bipolar, monopolar or even a lack of neurite growth. The goal of neuron culture is to induce these cells to regrow their neurites in an observable and quantifiable way (54). Thus, although the majority of neurons are bipolar *in vivo*, neurons with different morphologies may still be found *in vitro*. The dissociation, culture and detection of SGNs, and the consequent experiments involve delicate procedures that must be performed carefully and patiently.

Adenovirus vectors have been widely used in a number of fields (55,56). In the present study, an Ad-Epo was successfully constructed and transfected into the cultured SGNs. The results showed that Epo protein expression was markedly upregulated, indicating that Ad-Epo is a powerful tool for examining how Epo affects SGNs. At day 7 of culture, the rate of apoptosis was significantly higher in the vector control group compared with that in the Ad-Epo group, indicating that Epo may facilitate the survival of the cultured unimpaired SGNs and that apoptosis could be reduced using adenovirus transfection and other molecular biological techniques.

This study presented a thorough and precise description of Epo and Epo-R localization in the inner ear of neonatal rats using morphological and biomolecular methods. The expression pattern of Epo and Epo-R in normal neonatal

rats was visualized using immunohistochemistry and fluorescence staining, and Epo protein expression was also detected using western blotting. Additionally, it was found that Epo had a potential neuroprotective effect on normal cultured SGNs, as the rate of apoptosis was decreased by adenovirus vector-mediated upregulation of Epo expression. The results of this study may provide an experimental basis for a possible neuroprotective role of Epo based on down-regulation of the apoptosis rate in the mixed spiral ganglion cultures. Furthermore, the results of this study may add a novel dimension to our understanding of the complex picture of the neuroprotective effect of this clinically familiar drug and its possible mechanism of action in the inner ear.

Despite these findings, the following issues remain unclear: i) The exact pattern of Epo and Epo-R expression in the inner ears of neonatal rats; ii) the neuroprotective potential of Epo for SGNs in the inner ear; iii) the other cellular activities that are regulated by Epo; and iv) the mechanism by which Epo exerts its modulatory effects. Thus, further study is required to clarify these issues and to elucidate the protective role of Epo and its underlying mechanism. In the future, other intervention factors may be employed to cause damage to SGNs, or *in vivo* experiments may be performed with hearing-impaired individuals.

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Competing interests

The authors declare that they have no competing interests.

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