Transplantation of Neural Stem Cells into the Modiolus of Mouse Cochleae Injured by Cisplatin

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INTRODUCTION

Hearing impairment is the most frequent disability of people in industrialized countries. The loss of spiral ganglion neurons (SGNs) is a major cause for profound hearing loss (1). SGNs are auditory primary neurons in Rosenthal’s canal in the modiolus of the cochlea. Sound stimulation is adapted by auditory hair cells, converted into electronic stimulation in hair cells, and transmitted to SGNs. The survival of SGNs is therefore a critical issue for maintenance of hearing function, and for obtaining the clinical benefits of cochlear implants, which are implantable devices designed to stimulate SGNs electronically. Regeneration of SGNs has thus been an important issue for restoration of hearing because their regenerative activity is very limited (2). New experimental approaches including cell transplantation are, thus, being pursued to restore hearing (3–5).

The aim of this study was to examine the potential of cell transplantation for restoration of SGNs. Fetal neural stem cells (NSCs) that have the potential for differentiation into neurons (4–7) were used as donor cells, and adult mice affected by cisplatin, in which severe degeneration of SGNs is induced (8), were the recipient animals.

MATERIALS AND METHODS

The Animal Research Committee, Graduate School of Medicine, Kyoto University approved all experimental protocols. Animal care was under the supervision of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

Fetal mouse NSCs expressing green fluorescence were used as transplants (4, 5). NSC spheres were obtained from the neuroepithelium of the dorsal telencephalon of embryos at embryonic day 11.5 of C57BL/6Jtg14 (Act-EGFP) Obs-Y01 transgenic mice (9) using the neurosphere culture medium (10, 11). Secondary neurospheres, which exhibited expression of nestin, but no expression of neuron-specific class III beta-tubulin (TuJ1) or glial fibrillary acidic protein (GFAP), were collected, dissociated and suspended for transplantation at a density of $1 \times 10^5$ cells/µl in the neurosphere culture medium. C57BL/6 mice at 6 weeks of age ($n = 4$) were used as recipient animals. A cisplatin solution (2.5 mg/ml in physiological saline; Sigma, St Louis, MO, USA) was injected from the left posterior semicircular canal. The procedures used are described in detail elsewhere (12). In this model, about 60% of SGNs disappear at 14 days after cisplatin treatment (8). The left cochlea of recipient animals was exposed under anesthesia with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (9 mg/kg) 14 days after treatment of local application of cisplatin. The medium containing NSC aggregates (2 µl) was then injected into the cochlea through the round window toward the direction of the cochlear modiolus using a Hamilton syringe and infusion pump. The round window membrane was then covered with connective tissue and adherent agents.
On day 14 after transplantation, the left cochlea of recipient animals was re-exposed after administration of lethal doses of ketamine and xylazine, and 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) at pH 7.4 was gently perfused into the perilymph from the round window. The animals were then sacrificed by cervical dislocation. The temporal bones were collected and immersed in the same fixative for 4 h at 4°C. Cryostat sections 10 μm thick were made, and the mid-modiolus sections were used for histological analysis.

The cell fates of grafted NSCs were determined by immunohistochemistry for TuJ1, a marker for neurons, or GFAP, a marker of glial cells. Anti-TuJ1 rabbit polyclonal antibody (1:300; Covance Research Product, Berkeley, CA, USA) or anti-GFAP rabbit polyclonal antibody (1:200; Sigma) was used as the primary antibody, and Alexa Fluor 594-conjugated anti-rabbit or goat IgG (1:200; Molecular Probes, Eugene, OR, USA) was as the secondary antibody. Counterstaining with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) was performed for demonstration of nuclear chromatin. The specimens were viewed with a Nikon ECLIPSE E600 fluorescence microscope (Nikon, Tokyo, Japan).

The numbers of transplant-derived cells in the modiolus in one section and the ratios of positivity for each marker in transplant-derived cells were examined. Both EGFP- and DAPI-positive cells were defined as transplant-derived cells. The average of two sections for each animal was defined as the number for that animal. The ratio of positivity for each marker in transplant-derived cells in the modiolus was then calculated.

RESULTS

We confirmed robust survival of the injected cells in cochleae of all experimental animals. NSC-derived cells were located in the modiolus of cochleae or scala tympani (Fig. 1A). NSC-derived cells injected into the basal part of the modiolus were also found in the apical end of the modiolus, indicating the migration activity of NSCs in the modiolus of cochleae. Some NSC-derived cells settled in spiral ganglia. The mean and standard deviation of numbers of NSC-derived cells in the modiolus was 190.5 and 60.8.

NSC-derived cells in the modiolus exhibited expression of TuJ1 (Fig. 1B–D) or GFAP (Fig. 1E–G). TuJ1-positive grafted cells were predominantly located in the osseous spiral lamina or spiral ganglion. Expression of TuJ1 was observed in about 10% of NSC-derived cells. Immunohistochemistry for TuJ1 also demonstrated an apparent decrease of SGNs. The majority of NSC-derived cells were positive for GFAP (Fig. 1E–G). Expression of GFAP was observed in about 80% of NSC-derived cells. GFAP-positive grafted cells were found in the modiolus and spiral ganglion. Interestingly, TuJ1-positive grafted cells...
were positioned on the periphery of grafted cells (Fig. 2A), while GFAP-positive grafted cells were mainly located in the center of grafted cells (Fig. 2B).

**DISCUSSION**

Previous studies have demonstrated that NSCs grafted into the inner ear survive in the cochlear cavity of newborn rats (3) and adult mice (4). In addition, it has been demonstrated that NSCs grafted into the cochlear cavity migrate into the modiolus and sensory epithelia of cochleae damaged by aminoglycosides (5). These findings suggest that NSCs can be a source of transplants for cell therapy of degenerative inner ear diseases. It has also been revealed that grafted NSCs surviving in the cochlear cavity differentiate into neural cells in normal inner ears (4) and in injured inner ears (5), suggesting that NSCs can be transplants for restoration of SGNs. We thus used NSCs as transplants for restoration of SGNs in this study. In previous studies on NSC transplantation into the inner ear, injection of NSCs into the cochlear duct (3, 5) or semicircular canal (4) was used as procedures for transplantation. However, no or few NSC-derived cells in the modiolus have been identified in these studies. We thus used a direct injection into the modiolus as a method for NSC transplantation to introduce NSCs into the modiolus.

The present findings demonstrate the robust survival of NSC-derived cells in the modiolus. In addition, NSCs injected in the basal portion of the modiolus settled in the apical end of the modiolus and osseous spiral lamina, indicating the high potential of NSCs for migration. The migration capacity of NSCs has also been reported in the spinal cord (13). Therefore, an injection of NSCs in the basal portion of the modiolus is an effective method for introduction of NSCs into the modiolus from the base to the apex.

Immunohistochemistry for TuJ1 and GFAP revealed the cell fate of grafted NSCs surviving in the modiolus. NSC-derived cells differentiated into neurons in the modiolus, indicating that NSC transplantation into the modiolus may be utilized for restoration of SGNs. However, only 10% of NSC-derived cells differentiated into neurons, similarly to previous findings when NSCs are grafted into the fluid space of the inner ear (4, 5). On the other hand, it is noteworthy that TuJ1-positive grafted cells distributed on the periphery of the mass of grafted cells (4), while GFAP-positive ones are central (5). (GFP = green fluorescence protein.) Scale bar represents 30 µm.

Functional recovery of SGNs requires more abundant neurons derived from transplants. Therefore, promotion of the activity of neural differentiation or increase of numbers of surviving NSC-derived cells was crucial for functional recovery of SGNs. Neurotrophins reportedly stimulate neural differentiation of NSCs (14). In addition, several neurotrophins have effects for promotion of neurite outgrowth from NSCs (15). Therefore, the supplement of neurotrophins may be a cue for the promotion of the survival of NSCs grafted in the inner ear and functional recovery. On the other hand, the microenvironments where NSCs settled influence the fate of grafted NSCs. Cytokines associated with inflammation influence the survival and differentiation of NSCs transplanted in the nervous system (16). The microenvironments of the cochlea also alter depending on the time after traumatic treatment (8). Optimization of the timing of transplantation of NSCs is hence required for increased numbers of NSC-derived neurons in the modiolus.

In conclusion, an injection of NSCs into the modiolus of injured cochleae results in robust survival of grafted NSCs in the modiolus. Grafted NSCs differentiate into neurons in the modiolus, although their number is limited. NSC is a possible candidate of cell therapy for restoration of SGNs.
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