Original Article

Migration of R28 Retinal Precursor Cells into Cochlear and Vestibular Organs

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Abstract Damaged hair cells and neurons in the inner ear generally can not be replaced in mammals. The loss of these cells causes permanent functional disorders in both the cochlear and vestibular systems. Transplantation of retinal precursor cells, R28 cells, into inner ear tissue may help replace missing cells. The aim of the current project was to induce R28 cell transdifferentiation into cochlear and vestibular cell types under culture conditions. The first part was related to R28 cell labeling with DiI fluorescence that would help identify and track R28 cells. The second part involved co-culturing R28 cells in cochlear and vestibular organotropic cultures or isolated spiral ganglion neurons. The results suggest that R28 cells have the potential to differentiate into supporting cell types and spiral ganglion neurons in serum free medium, probably under the influence of diffusible signals from inner ear tissues. This information is useful for future efforts in inducing stem cell differentiation in the inner ear to replace lost sensory and neural cells.

Key words retinal precursor cells; cochlea; vestibule; spiral ganglion neuron; culture

Introduction

Permanent cochlear and vestibular damage can be caused by a number of factors including: ototoxic drugs, acoustic trauma, genetic disorders, aging, anoxia, viral infection, bacterial infection and more (Ding and Salvi, 2005; Ding et al, 1999, Salvi et al, 2001). Damaged hair cells and missing neurons cannot be replaced spontaneously because mammalian hair cells and neurons do not have regenerative capability. For this reason, hearing aids and cochlear implants are currently the only hope for severely hearing impaired patients to return to the auditory world, with multiple limitations. For patients to be able to use hearing aids, a certain level of residual hearing is required. In cochlear implant patients, post-operative training and establishment of effective signal transmission between the implant and the brain are necessary.

Stem cells are known for their ability to integrate into host tissues, and have been successfully used in several transplant experiments in the brain, spinal cord and eye to replace damaged neurons and sensory cells. In the inner ear, stem cell therapy has also been recently studied in mammals. Preliminary results have shown that transplanted stem cells are capable of migrating or grafting into cochlear and vestibular end organs and into neural ganglions(Hu et al, 2005, Iguchi et al, 2004; Tamura et al, 2004), indicating their potential in future treatment for inner ear disorders.

The R28 retinal precursor cell line was developed by Gail M. Seigel from P6 rat retinas, and immortalized with the 12S E1A gene from adenovirus(Seigel, 1996; Seigel, 1998). It is a stable cell line that has been used to characterize neuroepithelial and glial cells(Seigel, 1996). R28 cells have the potential behave as uncommitted central nervous system(CNS) progenitor cells, or neuroepithelial cells, as they bear concurrent expression of both glial and neuronal markers. Previous studies indicate that R28 precursor cells are able to migrate, integrate and differentiate into immunocompetent host tissues without forming tumors, and respond to cues in the surrounding environment. Importantly, R28 cells can be used for in vivo studies in non-immunosuppressed animals since the replication-incompetent vector used to immortalize the cells does not lead

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to infectious virus production. R28 cells have been widely used in research involving ischemia(Xu et al, 1999), autoimmune-mediated toxicity(Adamus et al, 1997, 1998), glaucoma drug testing(Tezel, et al, 1998), retinal transplantation, and apoptosis (Seigel and Liu, 1997). The ability of R28 progenitor cells to migrate, integrate and differentiate in the host environment depends on complex interactions between external signals including growth factors and neurotransmitters, as well as on receptors present on the host and grafted cells (Cameron and Mckay, 1998; Cameron et al, 1998; Sah et al, 1997). Since R28 progenitor cells are capable of migrating into a variety of tissues, it is reasonable to expect that they may also be able to migrate into inner ear tissues.

The current study focuses on determining if R28 cells are able to migrate into cochlear or vestibular tissues and to continue to proliferate and transdifferentiate into cell types of the host environment, using co-culture techniques with or without gentamicin-induced tissue damage.

Materials and methods

R28 retinal precursor cells: The Immortalized E1A-NR.3 cell line and its subclone R28 were developed through 12S E1A-induced immortalization of postnatal day 6 rat retina (Whyte et al, 1998). Immortalized cells were maintained in Dulbecco's Modified Eagle's Medium+ (DMEM+) with 10% calf serum, 1X MEM vitamins (GIBCO), 0.37% sodium bicarbonate, 0.058% L-glutamine, and 100 μg/ml gentamicin.

R28 cell labeling: Stock solution of fluorescent DiI (1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate) (Molecular Probes, Inc., Eugene, OR) was prepared in DMSO at 1-2mg/ml in advance. Before labeling, DiI stock solution was diluted directly using DMEM culture medium or serum free medium to a suitable working concentration at 1-2 μM, 1-2 μg/ml. R28 retinal precursor cells were incubated in the DiI working solution for 5 minutes at 37℃, and then an additional 15 minutes at 4 ℃. Incubation at the lower temperature allowed the dye to label the plasma membrane, while slowing down endocytosis, thus reducing dye distribution in cytoplasmic vesicles. After labeling, R28 cells were rinsed with calcium-magnesium-free PBS, and trypsinized with 0.0625% trypsin for 5 minutes until cells were isolated from the dish wall. Trypsinization was terminated by adding 10% calf serum. Cells were collected and centrifuged at low speed and suspended at a density of 5,000 cells per μl in DMEM culture medium or serum free medium. R28 cells were freshly labeled with DiI before transplantation in co-cultures with inner ear cells.

Spiral ganglion culture system: The head and neck area of postnatal rats was disinfected with 75% alcohol. Animals were then decapitated. The spiral ganglion neurons were harvested from the Rosenthal's canal and placed in Hanks' Balanced Salt Solution. Spiral ganglion tissue was incubated with 0.125% trypsin and 0.125% collagenase for 20 minutes at 37℃. Trypsinization was terminated by adding 1ml of a solution containing 150 μl fetal bovine serum and 850 μl DMEM. Spiral ganglion tissue was triturated by a fire-polished Pasteur pipette in 0.05% DNAse in Basal Medium Eagle(BME). The remaining undissociated tissue was separated by filtering with a nylon meshwork(33 μm pore size). Spiral ganglion suspension was seeded in individual culture dishes at a 200 cells/mm2 concentration on polylysine(500μg/ml) / laminin(20 μg/ml) coated plates. The culture medium was replaced every other day.

Cochlear and vestibular organotropic culture system: The organ culture procedures are similar to those described previously (Zheng and Gao, 1996; Ding et al, 2002). Two-day-old(P2) F344 rats were used in this study. Pregnant rats were obtained from the Charles River Laboratories. At P2, the postnatal rats were decapitated. The cochlear basilar membrane and macula of the utricle were carefully dissected. A 15μl drop of rat tail collagen was placed on a 35 mm culture dish(Falcon 1008, Becton Dickinson) and allowed to gel for approximately 15 minutes. Type I rat tail collagen (Collaborative Research, 3.76 mg/ml in 0.02 N acetic acid) was mixed with 10X BME and 2% sodium carbonate with a 9:1:1 ratio prior to use. Then 1 ml of serum free medium with BME plus serum free supplement (Sigma I1884, 1%), 1% bovine serum albumin, 2 mM glutamine, 5 mg/ml glucose and 10 U/ml penicillin were added to the culture dish. The cochlear basilar membrane and macula of the utricle were positioned on the collagen as a flat surface preparation by gently pressing on the tissue with forceps. Surface tension from the thin layer of culture medium helped to hold the tissue against the underlying collagen. The cochlear and vestibular explants were placed in an incubator(Forma Scientific 3029, 37°, 5% CO_2) overnight. On the second day, cultured tissues were divided into two groups. One group was cultured in a medium containing 3 mM gentamicin for 24 hours and then in a serum free medium for up to 12 days. The other group was cultured in the serum free medium throughout the en-