Original Article

Retrovirus-Mediated Gene Transfer in Immortalization of Progenitor Hair Cell Lines in Newborn Rat

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Abstract Objective To present an experimental method that allows isolation of greater epithelial ridge (GER) and lesser epithelial ridge(LER) cells from postnatal rat cochleae using a combinatorial approach of enzymatic digestion and mechanical separation and to investigate a retrovirus-mediated gene transfer technique for its possible utility in immortalization of the GER and LER cell lines, in an effort to establish an in vitro model system of hair cell differentiation. **Methods** GER and LER cells were dissected from postnatal rat cochleae and immortalized by transferring the SV40 large T antigen using a retrovirus. The established cell lines were confirmed through morphology observation, immunnocytochemical staining and RT-PCR analysis. The Hath1 gene was transferred into the cell lines using adenovirus-mediated techniques to explore their potential to differentiate into hair cells. **Results** The established cell lines were stably maintained for more than 20 passages and displayed many features similar to primary GER and LER cells. They grew in patches and assumed a polygonal morphology. Immunostaining showed labeling by SV40 large T antigen and Islet1(a specific marker for GER and LER). All passages of the cell lines expressed SV40 large T antigen on RT-PCR analysis. The cells also showed the capability to differentiate into hair cell-like cells when forced to express Hath1. **Conclusion** Retrovirus-mediated gene transfer can be used in establishing immortalized progenitor hair cell lines in newborn rat, which may provide an invaluable system for studying hair cell differentiation and regeneration for new treatment of sensory hearing loss caused by hair cell loss.

Key words retrovirus, immortalization, hair cell regeneration, progenitor cells, GER, LER

In the mammalian auditory system, cochlear hair cell loss resulting from noise, aging, and aminoglycoside toxicity is irreversible and leads to permanent sensorineural hearing loss. Although hair cell replacement occurs spontaneously following injury in birds and lower vertebrates[1, 2], replacing lost hair cells in the mammalian cochlea is currently unavailable. To restore hearing, it is necessary to generate new functional hair cells. One of the principal and feasible techniques for potential cell replacement in mammals is a renewable source of hair cell progenitors that can be transplanted into damaged inner ears. Recent work suggests that greater epithelial ridge (GER) and lesser epithelial ridge (LER) cells in the inner ear may serve as hair cell progenitors[3].

Studying the molecular and cellular mechanisms underlying the development and regeneration of hair cells has been hampered by difficulties in sample preparation due to small tissue size and complicate bony structures

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of the inner ear. Establishment of immortalized cell lines from hair cell progenitors can provide unlimited materials for moleculobiological, biochemical, and electrophysiological studies of hair cell progenitors.

In this report, we successfully isolated a pure population of hair cell progenitors including GER and LER cells from postnatal rat cochlea, which were transfected with the SV40 large T antigen oncogene using a retrovirus-mediated gene transfer technique for immoratalization. The established cell lines were stable through passages, displayed features similar to primary GER and LER cells, expressed SV40 large T antigen and Islet1, and showed the ability to differentiate into hair cell-like cells. The technique may provide an in vitro model system to study hair cell differentiation and regeneration.

Methods

Preparation of pure cochlear sensory epithelial cell cultures

To obtain cochlear epithelial sheets (CES), basilar membrane dissected from postnatal day 1 SD rats was incubated in 0.5 mg/ml thermolysin and 5 u/μl DNase in D-Hanks' solution for 30 minutes at 37℃, to facilitate separation between epithelium and connective tissue. Partially dissociated cochlear epithelial cell cultures were mechanically triturated using a pippett and then placed in a 96-well Costar culture plate in serum supplemented medium (Dulbecco's modified eagle media plus 10% fetal bovine serum and 10 units/ml penicillin).

Infection of cultures with retroviral constructs containing SV40 Large T antigen

After 1 day in culture, pure cochlear sensory epithelial cells were infected with conditioned medium collected from Φ2 cells packaging the retrovirus encoding large T[4] in the presence of 8 μg/ml polybrene (Sigma) and 20 ng/ml EGF[5] (Promage). After two infection sessions (5-6 hours each), the virus-containing medium was replaced with serum supplemented medium. Several days after infection, the cultures were lifted using diluted trypsin solution (0.125%) and passed to a new 24-well plate in the same culture medium. Clones of infected cells were collected at Day 10 with a cloning ring. The cell lines established were made up mainly of GER and LER cells, because retrovirus infected only the proliferation-capable GER and LER cells (in contrast to post-mitotic IHCs or OHCs). The cultures were expanded and passed several times before being stored in a freezer.

Identification of GLERC-6 cells

GLERC-6 cells were plated at a density of 3000-4000 cells/well in 24-well slides in regular serum supplemented medium at 33℃ for 1-2 days before fixation in 4% paraformaldehyde for 40 minutes. The preparations were blocked with phosphate buffered saline (PBS) containing 10% normal goat serum for 30 min and then incubated with monoclonal antibodies against large T antigen (1:100, Oncogen Science) and Islet1 (1:1000, Hybridoma Bank) in PBS containing 3% normal goat serum overnight at 4℃. DAB staining was used to examine large T antigen and Islet1expression. RT-PCR analysis was performed to test expression of SV40 large T antigen in different passages of GLERC-6 cells. The total RNA was extracted with Trizol from passages 6, 10 and 15, using dd H_2O and CES tissues as controls. Specific primers for SV40 large T antigen[6] (forward primer, GTTATGATAACTGTTATG; reverse primer, GAAATGCCATCTAGTGAT) were used.

Hath1 Gene Transfer

To determine if GLERC-6 cells were able to differentiate into hair cells, they were incubated with minimal volume of recombinant adenoviruses containing ad-Math1-EGFP and ad-EGFP (courtesy of Dr. Wei-Qiang Gao, Department of Neuroscience, Genentech, Inc., USA.) for 2-3 hours and then with serum supplemented medium for 8 days. Cultures were then fixed in 4% paraformaldehyde for 40 minutes before anti-myosin Ⅶa (1:100, Sigma) immunocytochemistry assay.

Results

Isolation of cochlea epithelial sheets

CES separation from the basilar membrane was easy after junction between the epithelium and cochlear connective tissue had been loosened through thermolysin incubation (Fig. 1A). The isolated CES included GER, IHC, OHC and LER and the spatial relationship among IHCs, OHCs GER and LER cells was clearly identified in the preparation (Fig. 1B).

Growth characteristics of primary cochlea epithelial cells