Neuroscience Letters 487 (2011) 383–388

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

In vitro effect of altering potassium concentration in artificial endolymph on apoptosis and ultrastructure features of olfactory bulb neural precursor cells

Menghang Wang, Jianhua Qiu*, Wenjuan Mi, Feng Wang, Juan Qu

Department of Otolaryngology, Xijing Hospital, Fourth Military Medical University, No.17, Changle West Road, Xi'an, 710032 Shaanxi Province, China

ARTICLE INFO

Article history:
Received 26 August 2010
Received in revised form 22 October 2010
Accepted 23 October 2010

Keywords:
Endolymph
Potassium concentration
Apoptosis
Ultrastructure
Olfactory bulb
Neural precursor cells

ABSTRACT

Transplantation of neural stem cells (NSCs) into the cochlea to replace irreversibly damaged sensory epithelia is a potentially valuable remedy for hearing loss. Several mammalian stem cell lines are being successfully transplanted into, or migrated to, the endolymph (EL) fluids environment of the cochlea. However, the survival rate of transplanted cells is relatively low. This study focused on the effect of altering the potassium (K^+) concentration of artificial EL on cell survival and apoptosis of olfactory bulb neural precursor cells (OB NPCs) *in vitro*. OB NPCs were prepared and placed in media for 24 h, supplemented either with artificial EL, or artificial EL-like solutions of different K^+ concentrations. Survival, apoptotic features and ultrastructural changes in the cells are noted. Artificial EL-like solutions, especially with K^+ concentrations of 50 mM or more, resulted in a series of necrotic or apoptotic events. Lower K^+ concentrations (30 mM) decreased apoptosis and necrosis, improving the survival rate of cultured NPCs. Thus, it is conceivable that the external K^+ concentration in EL is a key environmental factor to regulate the survival of exogenous stem cells.

In mammals, aminoglycoside antibiotics, noise and aging can induce irreversible damage to the sensory (hair) cells of the cochlea and lead to permanent hearing loss [1,2]. Stem cells transplanted into the cochlea to replace irreversibly damaged neurons or hair cells are a potential therapy for congenital and acquired deafness. Regeneration of the inner ear hair cells is thus, considered the ultimate therapy for hearing loss [3].

Several mammalian stem cell lines are being successfully transplanted *in vivo* into the cochlear fluids environment. The transplanted cells move to different positions in the inner ear, and survive and differentiate into a variety of cell types [4–7]. In addition, stem cells transplanted into the endolymph (EL) fluid environment can partially survive and differentiate into new cell types capable of expressing hair cell markers [8]. It was reported that olfactory bulb neural precursor cells (OB NPCs) were ideal source for cells to be transplanted into damaged regions of the nervous system and, *in vitro*, can be differentiated into hair cells [9–11]. Therefore, investigating the effect of features of the EL fluid environment on transplanted cells may provide a new avenue to restore hearing.

As an unusual extracellular fluid, the composition of EL is characterized by a high concentration of potassium ions (K^+). It is generally accepted that this high ion concentration has a major role in the processes of sensory transduction. It was reported that undifferentiated and partially differentiated embryonic stem (ES) cells delivered to the scala media of the inner ear were discovered near the spiral ligament and stria vascularis, able to survive in the high K^+ endolymphatic solution for at least nine weeks [8]. On the other hand, other reports are that ES cells are transplanted into the EL with low efficiency and survival rates [6,12], indicating that the cochlear microenvironment may be hazardous to transplanted progenitor cells. Moreover, there is a view that young and immature cells in the cochlea are particularly vulnerable to apoptosis, and EL may be a formidable challenge for exogenously transplanted neural stem cells (NSCs) and NPCs [13–15]. Some reports indicate that K^+ concentration and K^+ channels play an important role in the apoptosis of neurons or stem cells, and excessive K^+ efflux and intracellular K^+ depletion are involved in the early steps of the apoptotic cascade [16–18]. Therefore, altering the K^+ concentration in the EL microenvironment may provide insights into the survival and apoptosis of stem cells and precursor cells whose purpose is the regeneration of hair cells.

In this study, we prepared OB NPCs and cultured them for 24 h (h) in media which were supplemented either with artificial EL solution, or artificial EL-like solutions of different K^+ concentrations. Survival, apoptosis, necrosis and ultrastructural changes were observed.

* Corresponding author. Tel.: +86 29 84773427; fax: +86 29 83224744.
E-mail addresses: qiujh@fmmu.edu.cn, qiujh808@yahoo.com.cn (J. Qiu).

0304-3940/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2010.10.061

All animal procedures were approved by the Animal Welfare Committee of the Fourth Military Medical University. All animals were obtained from the animal center of the Fourth Military Medical University (Xi'an, People's Republic of China).

Healthy Sprague–Dawley (SD) rats weighing 250–300 g were bred, with embryonic day 0 (E0) defined as the day on which a vaginal plug was found. At day E12.5–14.5, rats were anesthetized under sterile conditions with 2% sodium pentobarbital (2 ml/kg; Sigma, USA) and decapitated. Embryonic brains were removed and the olfactory bulbs were dissected. NPCs were prepared following previously published procedures [9,19]. All cells were seeded and cultured in Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient Mixture (DMEM/F12; Invitrogen Corp., CA, USA), containing streptomycin (50 μg/ml, Sigma) and penicillin (50 U/ml, Sigma, USA).Cells growing as sphere aggregates (neurospheres) were passaged at 4–7 d by mechanical procedures and were plated at 5000/cells per square centimeter at each passage. NPCs were obtained from the neurospheres after at least two passages at 37 °C and 5% CO_2.The cell spheres were fixed in 4% paraformaldehyde and incubated at 4 °C overnight with monoclonal mouse anti-nestin (1:100; Chemicon), monoclonal rabbit anti-Musashi (1:100; Chemicon). Secondary antibodies conjugated with the fluorescent dye Cy3 (1:100; Sigma) were used to detect the primary antibodies.

Cultures were maintained at 37 °C and 5% CO_2. The growth medium for cells was changed to Knock out™ DMEM/F12 (Invitrogen Corp., CA, USA) for low osmolality and simple composition. The artificial EL medium was supplemented with the following (in mM): KCl, 126; NaCl, 1; $KHCO_3$, 25; $CaCl_2$, 0.025; $MgCl_2$, 0.025; K_2HPO_4, 1.4; mannitol (Sigma, USA), 25 [20,21]. For determining the response to different K^+ concentrations, the cultures were grown in the artificial EL-like media with K^+ at 5, 30, 50, 70 or 150 mM. The pH was adjusted to 7.3–7.4 and the osmolality was balanced to between 330 and 340 mOsm/kg (Fiske Micro-Osmometer Model 210). The control group was cultured in Knock out™ DMEM/F12 medium. The NPCs were added to the treatment and control solutions at a density of 10^5 cells/uncoated plastic flasks and cultured for 24 consecutive hours. Changes were then assessed.

A microtiter plate assay which uses the tetrazolium salt MTT (Sigma, USA) was used to quantitate cell viability and proliferation. Following the pretreatment, aliquots of 200 μl of NPC suspension (10^5/ml) were seeded to three 96-well plates in eight replicates. MTT solution (5 mg/ml) in aliquots of 20 μl were added to each well and incubated for 4 h in a humidified 5% CO_2 incubator at 37 °C, followed by low centrifugation. Then, the 200 μl of supernatant culture medium were carefully aspirated and 200 μl aliquots of dimethylsulfoxide (DMSO) were added to each well to dissolve the formazan crystals. Optical densities were read on a microplate reader (Biotek XS2, USA) at 570 nm as the reference wavelength.

Caspase-3 activity was assessed with the sequence DEVD (Caspase-3 Colorimetric Activity Assay Kits, Chemicon, USA) according to the manufacturer's instructions. Cells were collected at a density of 0.5–2 × 10^6 cells (1500 rpm 10 min). Then, cells were resuspended in 50–500 μl of chilled 1X cell lysis buffer, incubated on ice for 10 min and centrifuged for 5 min with a microcentrifuge (10,000 × *g*). The supernatant (cytosolic extract) was transferred to a fresh tube and put on ice. The assay mixture (20 μl 5X assay buffer + 10 μl Caspase-3 sample + 60 μl DI H_2O + 10 μl Caspase-3 substrate), buffer blank, and substrate blank were prepared in a 96-well plate and cultured for 1–2 h at 37 °C. Samples were analyzed using a microplate reader (Biotek XS2, USA) at 405 nm.

The apoptotic cells were determined by Annexin V-FITC/PI FACs assessment. Apoptosis of cells was measured using the Annexin V-FITC apoptosis detection kit (BD Bioscience) following the manufacturer's instructions and analyzed by flow cytometry using BD FACSAria cell sorter and BD FACSDiva software (BD Biosciences, San Jose, CA). This experiment was performed 3 times to obtain a mean ± standard error of the mean (SEM) ($n = 3$).

To determine the necrotic/late apoptotic cells, a nuclei staining method utilizing the DNA dyes Hoechst 33342 and Propidium Iodide (PI, Sigma, USA) was applied. After exposure, pretreated cells were trypsinized, washed with PBS, and stained with PI (5 μg/ml) and Hoechst 33342 (1 μg/ml) for 10 min at RT. After rinsing in PBS, coverslips were examined using an Olympus BX60 fluorescence microscope equipped with a C4742-95-10sc digital camera (Hamamatsu Photomics Norden AB, Solna, Sweden). Cells were counted, scoring at least 300 cells in 5 microscopic fields randomly selected on each coverslip. The experiments were performed in triplicate.

To analyze the ultrastructural changes, electron microscopy was applied. After culture, the cells were centrifuged, washed in PBS and fixed for 1 h in 2.5% glutaraldehyde and 0.1 M sodium cacodylate. The cells were then washed three times with 0.1 M sodium cacodylate, post-fixed in 1% osmium tetroxide for 2 h and incubated in 2% tannic acid for 30 min. The cells were dehydrated in a graded ethanol series, and embedded for sectioning in epoxy resin. After the contrasted stained with 2% uranyl acetate and 2.6% lead citrate (10 min at RT), the ultrathin sections were examined under a transmission electron microscope (JEM-2000EX; JEOM, Tokyo, Japan).

The difference between groups was determined with one-way analysis of variance (ANOVA) followed by Tukey's test using Statistical Package for the Social Sciences (SPSS) 13.0 (SPSS Inc.) software. Differences were considered statistically significant at $P < 0.05$.

NPCs were isolated from olfactory bulbs of E14.5 rats (Fig. 1A), and plated in cell suspension under tissue culture conditions exposed to bFGF and EGF. After 7 days in culture, the olfactory precursor cells developed into rounded sphere cells which were dividing and forming cell aggregates or spheres (Fig. 1B). After 14 days, the spheres increased in size as a result of additional cell division and aggregation (Fig. 1C). Immunocytochemistry analyses revealed that cell spheres derived from OB expressed nestin and musashi positive stain (Fig. 1D and E), markers for neuroepithelial stem cells and early neural stem cells, respectively. Hoechst 33342 stained these nuclei (Fig. 1F).

Then the NPCs were added to artificial EL medium and cultured for 24 consecutive hours. Concurrently, OB NPCs were divided into five groups and cultured under the conditions of 5 mM, 30 mM, 50 mM, 70 mM, or 150 mM K^+ concentrations in artificial EL-like solutions in order to determine the response to K^+ concentrations. Survival, apoptosis and necrosis of OB NPCs were observed.

Artificial EL caused a remarkable decrease in cell viability under normoxic conditions. The survival of NPCs was assessed based on measurement of the colorimetric transition of MTT to formazan (Fig. 2A). All groups of receiving the K^+-treatment decreased their cell viability, and K^+ at concentrations of 50 mM or more showed a remarkable reduction of cell survival. But cells cultured in 30 mM K^+ mediums show higher cell viability than other groups (Fig. 2B).

We also investigated the level of caspase-3 activity which is a downstream event of apoptosis in NPCs. As shown in Fig. 2C, the level of caspase-3 activity in artificial EL group was significantly increased in comparison with controls. After treatment with the different K^+ concentrations in artificial EL-like solutions, there was increased activity of caspase-3 in a concentration-dependent manner when K^+ concentrations were 50 mM and more (Fig. 2D).

Neuronal apoptosis was determined by Annexin V-FITC FACS-analysis. The apoptotic rate increased dramatically in artificial EL cultures (Fig. 2E). In different K^+ concentrations in artificial EL-like solution groups, results showed that increase in late apoptotic cell rate was concentration-dependent when K^+ concentration was 50 mM and more (Fig. 2F). Cells cultured in 150 mM K^+ medium showed a higher late apoptosis and necrosis rate respectively (72.4 ± 6.37% and 8.1 ± 0.76%).