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ORIGINAL ARTICLE

Nestin Expression in Proliferating Cells of Cultured Human Vestibular Organs

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Objective; In studies of the sensory epithelium of vestibular organs, regeneration of hair cells was observed. Regeneration capability of hair cells may be considered to suggest the presence of endogenous stem cells that could regenerate damaged hair cells in these organs. Therefore, we tried to show that human vestibular sensory epithelium contains cells that display proliferating characteristics in these cultured organs.

Materials & Methods; We obtained sensory epithelia of utricle, saccule and 3 semicircular canals from the patient with vestibular schwannoma who underwent tumor removal via translabyrinthine approach. The cells were cultured in a medium with epidermal growth factor (EGF) and fibroblast growth factor (FGF). Proliferating cells were checked with bromodeoxyuridine (BrdU) and nestin.

Results; In the culture media of the macular utriculi, the macular sacculi, and the ampulla of semicircular canal, although a very small number, cells showed positive expression of BrdU and nestin. But we could not subculture and differentiate these cells into organ-specific differentiated cells, as hair cells and supporting cells.

Conclusion; It is thought that proliferating cells with nestin expression are present in the sensory epithelium of human vestibular organs.

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Hair cells are present in the organ of Corti in cochlea, the ampulla of semicircular canal, the macula utriculi and the macular sacculi in the vestibule. According to the performed studies, the regeneration of hair cells was observed in the sensory epithelia of some vestibular organs. However, spontaneous regeneration has not been observed in diseases associated with hearing loss caused by aging [1, 2]. Regeneration capability of hair cells in the vestibular organs may suggest the presence of proliferating endogenous cells (sensory progenitor cell) that could regenerate damaged hair cells in these organs.

In 2003, Li et al.[3] succeeded in separating and culturing cells with the characteristics of stem cells from sensory epithelium in the utricle of adult mammals. They were able to do the self-renewal, and form the sphere with inner ear sensory cell markers and neural stem cell markers. And they could also differentiate into hair cells and supporting cells. This could be considered that some part of the sensory epithelium in the utricle has the characteristics of stem cells, and in addition, they could differentiate into mature hair cells.

Therefore, we thought that we would be able to isolate and culture endogenous stem cells from the utricle in adult mammals, although it has not been reported in human vestibular organ. So we tried to isolate and culture the cells which have the proliferating capacity like stem cells from the sensory epithelia of human vestibular organs like the utricle, saccule, and semicircular canal. And those tissues were obtained from patient with a vestibular schwannoma during surgery.

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Materials & Methods

In a 60-year-old female patient, the macular utriculi, the macular sacculi and three ampullae of semicircular canals were obtained during the translabyrinthine approach to remove vestibular schwannoma. The written informed consents were obtained from the patient. The local Institutional Review Board of Kangnam St. Mary's Hospital approved this study.

Isolation and culture of cells

The obtained tissues were transferred to a tube containing Dulbecco's modified Eagle's media (DMEM, Gibco, Carlsbard, CA, USA), and washed 3 times with DMEM. Afterward, they were transferred to a 15 ml tube containing 0.25% trypsin (Gibco, Carlsbard, CA, USA), and incubated at 37°C for 20 minutes. Subsequently, after adding DNase (Invitrogen, Carsbard, CA, USA), the cells were triturated and incubated at room temperature for approximately 2 minutes to allow relatively big cells to precipitate on the bottom.

The supernatant were transferred to a new tube. The enzyme action was terminated by adding 10% fetal calf serum (Gibco, Carlsbard, CA, USA), and it was centrifuged at 1,000 rpm for 5 minutes. The pellet was dissolved in the culture medium of the mixture of F12 and DMEM at the 1:1 ratio containing B27 supplement (Invitrogen, Carlsbad, CA, USA), L-glutamin (Sigma, St. Louis, MI, USA) and gentamicin (Invitrogen, Carlsbad, CA, USA). During the initial culture, epidermal growth factor (EGF, Invitrogen, Carlsbard, CA, USA) and fibroblast growth factor (FGF, Invitrogen, Carlsbad, CA, USA) were added to the culture medium. Approximately half of the culture medium was changed to the new culture medium every 3 days, and at that time, each growth factor was added again.

(BrdU) Bromodeoxyuridine Labelling and Immunofluorescence Stain

At one week of the culture, to assess proliferating cells, 10 μM 5'-Bromo-2-deoxy-uridine (BrdU, ABD Serotec, Raleigh, NC, USA) was added to culture media for 24 hours. The cultured cells were grown in 6-wells covered with cover slides, and by a conventional histological fixation method, fixed with 4% paraformaldehyde for 15 minutes, and washed with PBS. After incubation with 0.2% triton-X100 PBS for 5 minutes, for BrdU staining, the cells were incubated with 2M HCl for 1 hour, and thus DNA denaturation was performed. Afterward, neutralization procedure for 5minutes was followed. And the cells was incubated with 0.1M Na2B407, and washed with PBS. Subsequently, blocking was performed using 10% goat serum.

For double staining with BrdU, the specific antibody to neural stem cell marker, nestin (Chemicon, 1:200) was diluted with PBS containing 10% goat serum. It was incubated at 4 0C for 12 hours, and washed with PBS. Subsequently, as the cyanine-conjugated secondary antibody, goat anti-rat IgG antibody (Alexa Fluor-red color, ABCam, Cambridge, UK, for BrdU) and sheep anti-mouse C2 (Cy2-green colour, Jackson Immunores. Baltimore, PA, USA, for nestin) were diluted with PBS containing 10% goat serum to 1:200 and added. After washing with PBS, it was mounted using the mounting medium for fluorescence (Vector Lab. Peterborough, UK). Afterward, 4,6-diamidino-2-phenylindole (DAPI, Vector Lab. Peterborough, UK) was used to stain the nucleus.

Results

In culture media of the sensory epithelia of 3 types of vestibular organs which are the macular utriculi (Fig. 1) , the macular saculi (Fig. 2), and the 3 ampullae of semicircular canals (Fig.3), although a very small number, the cells showed expression of BrdU which represents cell proliferation. By staining these cells with nestin, that is the marker of the neural stem cell, all the cells that showed positive reaction to BrdU expressed nestin. Among the cells that expressed theses two markers, those from the macular saculi made the sphere form or a cell colony pattern that have been thought to be the characteristics of neural stem cells. But the cells from the ampulla of semicircular canal and the macular utriculi, appeared in diverse

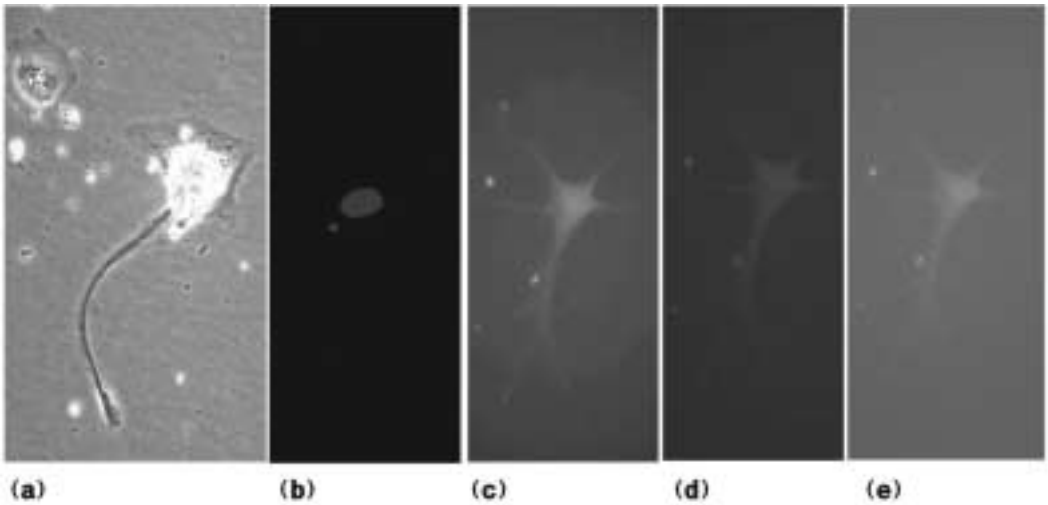

Figure 1. Cultured cell from human utricular macula at day 7. (X200)
(a) No stain
(b) Nuclear staining by 4,6-diamidino-2-phenylindole (DAPI) in blue
(c, d) Double staining for BrdU and nestin. A cell expressed stem cell markers, nestin (green) and BrdU (red). And this cell co-expressed both markers (e, merged).

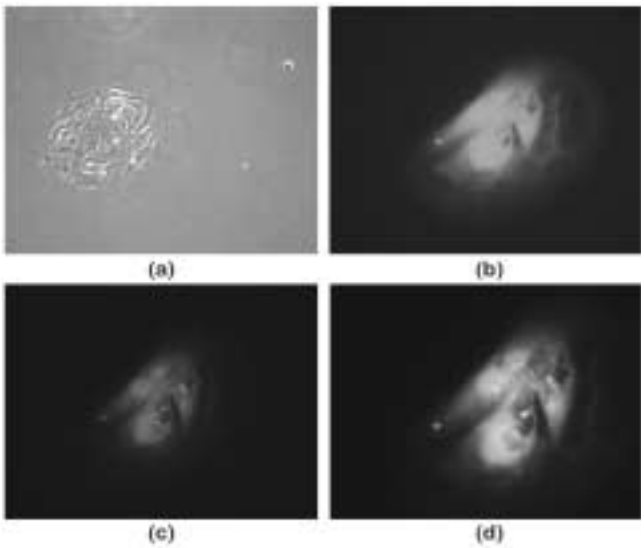

Figure 2. Cultured cells from human saccular macula at day 7. (X100)
(a) No stain
(b) Nestin positive cells were shown as green color.
(c) BrdU was expressed as red and nuclear staining was shown as blue by 4,6-diamidino-2-phenylindole (DAPI).
(d) Cells co-expressed both markers, BrdU and nestin (merged).

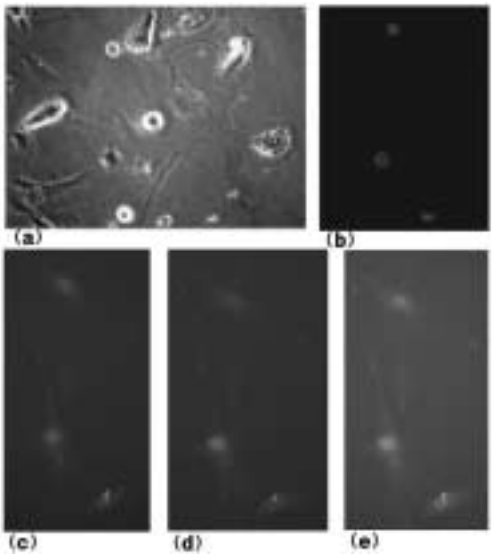

Figure 3. Cultured cells from human saccular macula at day 7. (X100)
(a) No stain
(b) Nestin positive cells were shown as green color.
(c) BrdU was expressed as red and nuclear staining was shown as blue by 4,6-diamidino-2-phenylindole (DAPI).
(d) Cells co-expressed both markers, BrdU and nestin (merged).

shapes depending on each organ. In the culture media from 3 ampullae of semicircular canals, a relatively larger number of cells were observed than the macular utriculi and the macular sacculi. The number of expressing both marker is less than 10, about [3,4] in utricle and saccule and about [7,8] in semicircular canals. It may be reason that ampulla of semicircular canal culture have 3 tissues from the 3 ampullae.

Discussion

Concerning the therapy using adult stem cells, good results were reported in vascular and hematological diseases. Recently, the presence of endogenous stem cells in each organ of the body in addition to stem cells in the umbilical cord blood and the bone marrow has been reported [2,4]. And it is known that organs with regeneration capacity such as the brain, skin, breast,

eye and gastrointestinal tract have endogenous stem cells in their organs. In addition, such adult stem cells can trans-differentiate into neurons or muscle cells, and thus the possibility of their use for the treatment of inner ear diseases has been implicated.[5,7]

On the other hand, endogenous stem cells were detected in the vestibular and auditory organs. The possibility of the self regeneration of cells in the inner ear is known and the studies using these stem cells have been ongoing actively.

At the organ of Corti of newborn rats, the presence of stem cells was confirmed, although it was not confirmed in adult mammals [8]. In addition, in the spiral ganglion of the inner ear of human and experimental animals, the presence of adult neural stem cells that had inner ear-specific characteristics and could undergo self-renewal and differentiate into neurons and Schwann cells has been confirmed [9,10].
And it has been reported that in the damaged vestibular organs, although partially, the regeneration of new hair cells occurs. Li et al. [3] proved the presence of adult stem cells in sensory epithelium of the macular utriculi of adult mouse. Similar to other stem cells, these cells have the capacity of self-renewal and form the spheres with the ability to differentiate into other cells. Neurons generated by the differentiation from these spheres expressed Pax-2, bone morphogenic protein (BMP-4) and BMP-7 that are specific to the developing stage of the inner ear. And these expressed transcription factors increased the hair cell markers, myosin VIIA and Brn3.1, on differentiated cells. In addition, the isolated and cultured cells forming the sphere were pluripotent, and in vitro as well as in vivo experiments, they were able to differentiate into the ectodermal neuronal cells and glial cells as well as the endodermal tissues such as the heart, the liver, the kidney, etc., and the mesodermal cells such as muscles. This implies the presence of endogenous stem cells in the sensory epithelium of the utricle of adult mouse. They have the characteristics of stem cells, and their differentiation into organ-specific cells was confirmed. Therefore, the regeneration by endogenous stem cells in the ear may be possible [7, 11,12].

But there is no report about the endogenous stem cells in human vestibular organ until now. It may due to the rarity and difficulty of sampling the human of the vestibular organ. So we tried to isolate and culture of proliferating and regenerative cells from the human vestibular organs. In this study, we used the markers BrdU and nestin for confirming the proliferating and regenerative cells.

Most of researches use these markers, such as BrdU and nestin for isolation and culture of neural stem cells. BrdU is a thymidine analog that incorporates DNA of dividing cells, in the S-phase of the cell cycle. BrdU is used for monitoring cell proliferation and confirming neurogenisis. Currently, BrdU labeling is the most widely used technique for studying adult neurogenesis [13]. Nestin is intermediate filament protein and expressed in neural stem cells. It is known as neural stem cell marker as with Sox and frizzled [14, 15].

We could find the cells expressing both markers simultaneously, although the number of cells was very few. It does not mean that they are stem cell, but it has meaningful result that human vestibular organs may have proliferating cells which regenerate damaged hair cells in certain condition.

Although we could not demonstrate the cells differentiated from the proliferating cells, it would be possible to isolate and culture spheres from human vestibular organ if we develop more appropriate culture conditions and have more human tissues.

This could show the possibility of cell therapy using organ-specific adult stem cells, which would avoid ethical problems pertinent to embryonic stem cells. In addition, attempts to regenerate cells by growth factors or neurotrophins may result in good outcome together with stem cell therapy. It has been reported that gene therapy using Math 1 gene could achieve the regeneration of sensory hair cells in the inner ear and its functional recovery [16, 17].

In this study, culture of the sensory epithelia of human vestibular organs, although a very small number of cells, has proliferating cells with expression of nestin.

However, it is thought that supplementary studies on the differentiation into specific cells that corresponds to the characteristics of stem cells and on the self-renewal are warranted. In addition, it is thought that acquiring more appropriate culture conditions for these cells is important.

Finally, we can conclude that in the sensory epithelium of human adult vestibular organs, proliferating and neural stem cell marker-expressing cells are present. And it implies that cells with the characteristics of adult neural stem cells could be present not only in the macula utriculi but also in the ampulla of semicircular canal and the macula sacculi.

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