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The effect of transplantation of nasal mucosal epithelial cell sheets after middle ear surgery in a rabbit model

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ABSTRACT

Postoperative regeneration of the middle ear mucosa and pneumatization of the middle ear cavity are of great importance after middle ear surgery. This study developed a new method to transplant autologous nasal mucosal epithelial cell sheets into the damaged middle ear cavity. The aim of this study was to evaluate postoperative healing after the transplantation of the cell sheets. Rabbit nasal mucosal epithelial cell sheets were fabricated on a temperature-responsive culture dish, and transplanted into the damaged middle ear of rabbit, which was surgically created. The healing of middle ears was evaluated by histology and X-ray computed tomography after transplantation. Functional evaluation was performed by measuring the maximum middle ear total pressure reflecting a trans-mucosal gas exchange function. Two control groups were used: the normal control group and the mucosa-eliminated control group. Transplantation of cell sheets suppressed the bone hyperplasia and the narrowing of pneumatic space in the middle ear cavity compared with the mucosa-eliminated control group. The mucosal gas exchange function was also better in the cell sheet-transplanted group. Nasal mucosal epithelial cell sheet was confirmed to be useful as an effective graft material after middle ear surgery and hopefully become a novel therapy in the future.

1. Introduction

In many cases with chronic otitis media including cholesteatoma and adhesive ottis media, tympanoplasty with mastoidectomy is presently a common surgical treatment. It is important to preserve normal middle ear mucosa as much as possible during surgical treatment for intractable chronic otitis media including cholesteatoma. The gas exchange function of middle ear mucosa plays an important role in pressure regulation of the middle ear cavity [1–6]. From the viewpoint of keeping good sound conduction, the maintenance of middle ear pressure is one of the most important functions of the middle ear mucosa. However, in the most patients with cholesteatoma, the extent of the lesion is so severe that the preservation of the middle ear mucosa is difficult. In a surgical treatment such as mastoidectomy, of which purpose is lesion removal, the removal of severe lesion results in the exposure of the bone surface and a poor blood circulation. Moreover, the postoperative regeneration of the middle ear mucosa is delayed, and the clinical functions are suppressed. This situation causes a renewed retraction and adhesion of tympanic membrane and the recurrence of cholesteatoma. Therefore, postoperative middle ear mucosal regeneration is of great importance after otological surgery.

If middle ear mucosa can be preserved and the rapid postoperative regeneration of mucosa on the exposed bone surface can be achieved after middle ear surgery, surgical treatment for otitis media including cholesteatoma can be potentially improved, and the physiological function of middle ear can be recovered. As a method to prevent a renewed retraction of the tympanic membrane from the attic and the recurrence of the cholesteatoma and adhesive otitis media, the use of oral mucosa, collagen sponge, or silastic sheeting have been reported [7,8]. However, the feasibility of these methods is hardly established.

In a previous study, our laboratory has developed artificial middle ear mucosa for promoting mucosal regeneration after middle ear surgery [9,10]. However, there are many residual problems and

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ethical issues before this method can be applied to actual human clinical application. Therefore, in considering the clinical application to actual humans in the future, this study developed a new approach to promote mucosal regeneration after middle ear surgery. Using temperature-responsive culture dishes [11], transplantable nasal mucosal epithelial cell sheets were fabricated, and the efficacies of the cell sheets were evaluated as a wound healing enhancing device after middle ear surgery in a rabbit model.

2. Materials and methods

2.1. Fabrication of nasal mucosal epithelial cell sheets

All experiments on animals were carried out in compliance with the Jikei University School of Medicine's guidelines for animal experiments. Mature domesticated rabbits (New Zealand white, 2–2.5 kg) were used in this study. These animals were confirmed to have neither otitis media nor other middle ear-related diseases. Each animal was painlessly and spontaneously anesthetized by the intravenous injection of pentobarbital (30 mg/kg). Using a surgical drill, a hole with 1 cm diameter was made in the surface of nasal cavity, and nasal mucosal tissue was surgically harvested, then, washed with Dulbecco's phosphate-buffered saline (PBS), and minced as finely as possible. The tissue was put on a culture dish coated with type I collagen (BD BioCoat, Franklin Lakes, NJ, USA), and subjected to primary explant culture in keratinocyte growth medium (KCM) containing 5% fetal bovine serum (FBS), hydrocortisone (0.5 μg/mL), insulin (5.0 μg/mL), transferrin (10 μg/mL), triiodothyronine (6.5 ng/mL), epidermal growth factor (0.5 ng/mL), cholera toxin (1 nM), penicillin G sodium (100 U/mL), and streptomycin sulfate (100 mg/mL). After 2 weeks, cultured cells were trypsinized, and the obtained cells were seeded on a temperature-responsive cell culture insert (CellSeed, Tokyo, Japan) at a density of 10×10^4 cells/cm^2. After a 10-day subculture in KCM, fabricated nasal mucosal epithelial cell sheets were harvested from the temperature-responsive cell culture insert by reducing temperature from 37 to 20 °C for 30 min.

2.2. Histological and immunohistochemical analyses of the nasal mucosal epithelial cell-sheets

For cross-sectional observations, native middle ear mucosa, native nasal mucosa, and harvested nasal mucosal epithelial cell sheets were fixed with 10% neutral buffered formalin, and routinely processed into 3 μm-thick paraffin-embedded sections. Hematoxylin and eosin (HE) staining was performed by conventional methods. For immunohistochemistry, de-paraffinized sections were washed with PBS and digested with protease K (DakoCytomation, Glostrup, Denmark). Sections were then treated with each of the following antibodies; mouse monoclonal anti-pancytokeratin (1:20 dilution, AE1/AE3, Abcam, Cambridge, UK), mouse monoclonal anti-vimentin (1:100 dilution, V9, DakoCytomation), mouse monoclonal anti-E-cadherin (1:100 dilution, NCH-38, DakoCytomation), according to the manufacturer's suggested protocol.

2.3. Transplantation of autologous nasal mucosal epithelial cell sheets

Mature New Zealand white rabbits were intravenously injected with pentobarbital. Using a microscope and a surgical drill, a small hole with 5 mm diameter was made in the lower surface of middle ear bulla, and the middle ear mucosa was eliminated as much as possible from the inferior edge of the tympanic membrane to the tympanic orifice of eustachian tube. Then, the autologous nasal mucosal epithelial cell sheets were transplanted into the middle ear bulla from which mucosa had been removed. Cell sheets were placed directly onto the bone surface using overlying polyvinylidene difluoride support membranes. Then, the animals were allocated into the following three groups; the normal control group ($n = 11$) of which animals had no surgical procedure, the mucosa-eliminated control group ($n = 7$) of which middle ear mucosa was removed with no transplantation, and autologous nasal mucosal epithelial cell sheet transplanted group ($n = 6$). Transplantable autologous nasal mucosal epithelial cell sheets were fabricated at 24 days after the removal of nasal mucosa. The fabricated cell sheets were transplanted into middle ear bulla, where the mucosa had been removed. Eight weeks after the transplantation, all animals were deeply anesthetized with over-dose pentobarbital for euthanasia and decapitated. For each group, X-ray computed tomography (CT) and histological analyses were performed, and the maximum middle ear total pressure (maximum METP) was measured as a functional evaluation. One-way analysis of variance (ANOVA) was used to confirm statistically significant differences among three groups. Turkey–Kramer test was used to correct for multiple comparisons when statistical significance was found in ANOVA. p-values less than 0.05 (P) for null hypothesis were considered statistically significant.

2.4. X-ray computed tomography after transplantation

Data acquisition was performed by with a CT scanner (LaTheta LCT-200, Aloka, Japan). Slices of 120 μm thickness were made from the temporal bones. The image data were subsequently quantified using an automated image analysis system. A slice containing attic, bulla, external ear canal, cochlea, and ossicles was used for evaluation. The capacity of each middle ear bulla was also measured.

2.5. Histological analyses after transplantation

Eight weeks after the transplantation, all animals were deeply anesthetized with over-dose pentobarbital for euthanasia and decapitated. The temporal bones were taken out from heads, fixed with 10% formalin solution, and decalcified for 2 weeks using a decalcifying solution. The sample was embedded in paraffin and first cut vertically along a line passing through the eustachian tube and the external canal. Then, thin sections were cut parallel to this cut surface. A section containing attic, bulla, and external ear canal was used for evaluation, and stained with Masson trichrome.

2.6. Post-transplantation functional evaluation of middle ear cavity

Functional evaluation was carried out by measuring the maximum METP as previously described [12,13]. Changes in the middle ear total pressure and its maximum value accompanying the trans-mucosal gas exchange of the middle ear mucosa have been reported to reflect the status of the mucosa [12]. Before the animals were euthanasia and decapitated, the measurement was performed on the animal under pentobarbital anesthesia and breathing spontaneously. First, a median incision was made in the soft palate, and then the eustachian tube was plugged by inserting a laminaria into the pharyngeal orifice of the eustachian tube, and this procedure gave no leakage of air. Next, a perforation was made in the tympanic membrane via the external ear canal. Then, a 10 cm-tube for gas exchange was inserted into the middle ear cavity, and a pressure sensor probe was placed in the external ear canal. The maximum pressure value was recorded. The maximum METP in normal rabbits was formed to be 6.35 ± 1.87 mmH2O (mean ± SD) [13].

3. Results

3.1. Fabrication of transplantable nasal mucosal epithelial cell sheets

Excised nasal mucosa was subjected to epithelial cell isolation. Primary epithelial cells grew and moved outside from the explants at 3 days after culture (Fig. 1A). Migration of epithelial cells from the periphery of nasal mucosal tissue fragments placed onto type I collagen-coated dishes and the quick propagation were observed. These cells showed a polygonal cobblestone shape, a typical morphology of epithelial cells (Fig. 1B). After 2-week culture, the outgrown cells were harvested by trypsin treatment, and seeded on temperature-responsive cell culture inserts at a density of 10×10^4 cells/cm^2. The cells were observed to have a steady growing ability with maintaining their cobblestone morphology even after 2 passages (Fig. 1C). After a 10-day subculture, all the cells reached successfully to confluent cell sheet and harvested as a contiguous transplantable cell sheet from the temperature-responsive cell culture insert by reducing temperature from 37 to 20 °C for 30 min (Fig. 1D).

3.2. Histology and immunohistochemistry

Immunohistochemically stained native middle ear mucosa and native nasal mucosa are shown in Fig. 2. The epithelia of both mucosa had a monolayer structure, but native nasal mucosal epithelium consisted of pseudostratified ciliated epithelium. Nasal mucosa had many glands in the subepithelial layer, but middle ear mucosa had no gland. These were large differences between middle ear and nasal mucosa. The expression patterns of pan-cytokeratin, vimentin, and E-cadherin of both mucosa were found to be similar. HE-stained specimens showed that fabricated cell sheets consisted multi-layered mucosal epithelial cells. Immunohistochemistry also showed that all epithelial cell layers of fabricated cell sheets expressed pan-cytokeratin. Vimentin showed a weak expression in the basal layer side of the cell sheet. Keratinocytes are known to express E-cadherin, which are cell–cell adhesion molecules found in adherens junctions. To examine the presence of cell–cell junctions in the fabricated cell sheets, immunohistochemistry for E-