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Galectin-7 as a Marker of Cholesteatoma Residue and Its Detection During Surgery by an Immunofluorescent Method—A Preliminary Study

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Objectives: To visualize the distribution of galectin-7 in middle ear cholesteatomas using an immunofluorescent method and to establish whether galectin-7 can be used as a marker of cholesteatoma residue at the time of operation.
Methods: Middle ear cholesteatomas were obtained at surgery from 30 patients. Samples were frozen and preserved in a freezer until histological study. After serial sectioning with a cryostat, 2 of the specimens were processed with primary antibody and Zenon rabbit immunoglobulin G labeling kits. After sufficient reaction time, the samples were observed using a confocal laser microscope. In the remaining 28 specimens, the cholesteatoma was treated as 1 block and stained with the same solution. It was then observed using a fluorescent stereomicroscope.
Results: Confocal microscopic analyses showed that galectin-7 was distributed in the cholesteatoma matrix. Because this area strongly stained green, it was easily recognized using a confocal laser microscope. In the stereomicroscopic study using the 1-block specimen in which the cholesteatoma was processed together with the surrounding granulation and mucosal tissue, only the matrix and overlying debris was yellow-green in response to excitation by light; the surrounding granulation and mucosal tissues did not respond in 7 specimens. In the remaining 21 specimens, the whole sample was composed of cholesteatoma and responded well to excitation by light. These findings suggest that galectin-7 might be a useful marker of cholesteatoma residue that can be visualized using this immunofluorescent method.
Conclusion: Because residual cholesteatoma matrix is considered to be one of the main causes of cholesteatoma recurrence, staining with galectin-7 at the time of operation would be a promising way to facilitate complete removal of the residue.
Key Words: Cholesteatoma recurrence—Confocal laser microscope—Fluorescent stereomicroscope—Immunofluorescent staining—Lectin family.
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In the treatment of middle ear cholesteatoma, complete removal of the matrix is the most important factor for preventing recurrence. However, this is sometimes difficult because cholesteatomas often extend deep into the middle ear cavity: cholesteatomas that extends into the tympanic sinus, oval window, or round window niche are notoriously difficult to access. In cases of open-type cholesteatoma, the matrix is hard to recognize when the overlying debris is missing. Even a small-sized residue might grow to be a large recurrent cholesteatoma. Recurrence can occur even after operation by expert surgeons (1–4).

At present, eradication of the matrix depends on the operator's experience and skill. To reduce the incidence of recurrence, it is indispensable to develop a new method that enables visualization of cholesteatoma residue at the time of operation. Using a proteomic analysis of human cholesteatoma, we found that galectin-7, which is a member of the lectin family, is a specific protein that is characteristic of the cholesteatoma matrix in the middle ear (5).

The aims of this article were to describe the distribution of galectin-7 in a removed specimen of human cholesteatoma using an immunofluorescent method and

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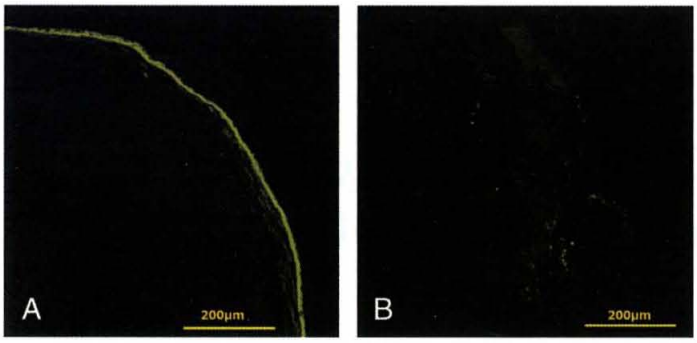

FIG. 1. Confocal laser microscopic findings of human cholesteatoma matrix (*A*) and middle ear mucosa (*B*) stained using an immunofluorescent method. Note that cholesteatoma matrix was strongly stained green, suggesting the presence of galectin-7, but such expression was never noted in middle ear mucosa.

to establish whether galectin-7 can be used as a marker of cholesteatoma residue at the time of operation.

MATERIALS AND METHODS

This study was conducted in accordance with the ethical committee of Ehime University Hospital and with the Helsinki Declaration. Before surgery, the purpose, method, and risks of the study were explained to the patients and their informed consent was obtained. Specimens were obtained from 30 patients with middle ear cholesteatoma (attic cholesteatoma in 20 cases, sinus cholesteatoma in 4 cases, congenital cholesteatoma in 1 case, and others in 5 cases). Immediately after removal, the specimen was embedded in OCT compound, frozen with hexane, and kept in a freezer at −80°C until histological study.

Two specimens were used to investigate the distribution of galectin-7 in cholesteatoma. After serial sectioning with a cryostat, these specimens were processed with primary antibody (rabbit monoclonal anti–galectin-7 antibody; Epitomics, Burlingame, CA, USA) and then with Zenon rabbit immunoglobulin G labeling kits (Invitrogen, Carlsbad, CA, USA) diluted 500-fold with phosphate-buffered saline, as Zenon's kits bind to the Fab fragment of the primary antibody. The reaction time was 60 minutes, according to the company's recommendation. In the present study, reaction times of 30 or 10 minutes were also tested in 1 specimen to investigate whether recognition of galectin-7 is possible with a shorter reaction time. After completion of staining, the specimen was observed with a confocal laser microscope (Nikon A1, Tokyo, Japan; spectrum of excitation light, 488 nm). An absorption test and positive control staining test to probe possible cross-reactivity with other members of the galectin subfamilies were not performed in this study because specificity of the monoclonal anti-galectin-7 antibody was already validated by Epitomics using Western blot analysis.

In the remaining 28 specimens, the cholesteatoma was treated as 1 block and stained with the same solutions for 10 minutes. The specimen was then observed with a fluorescent stereomicroscope (Leica MZ-FLIII, Wetzlar, Germany). This study was performed because, when the present method becomes clinically applicable in the future, the staining solution would be applied in the middle ear cavity to visualize possible cholesteatoma residue. In such situations, the residue can be detected by a surgical microscope (OPMI Pentero, Carl Zeiss, Göttingen, Germany) with laser illumination, which is already available for clinical use in neurosurgery.

RESULTS

The confocal microscopic study showed that galectin-7 strongly fluoresced green when exposed to excitation light. As shown in Figure 1, galectin-7 was expressed in cholesteatoma matrix that was composed of several epidermal layers. Although a little weak, the cholesteatoma debris also stained green, resembling sporadic green cotton dust inside the matrix. In contrast, granulation and middle ear mucosa did not show any response to excitation light. Figure 2 shows the findings when the reaction time was shortened. Apparently, a reaction time of 30 or 10 minutes was sufficient to recognize cholesteatoma matrix, although 60 minutes of staining showed the best image.

In the stereomicroscopic study using the 1-block specimen, in which the cholesteatoma was processed together with the surrounding granulation and mucosal tissue, only the matrix and overlying debris responded yellow-green to excitation light; the surrounding tissues did not respond in 7 of 28 specimens (Fig. 3). In the remaining 21 specimens, the whole sample was composed of cholesteatoma matrix that responded well to excitation light. The response was the same irrespective of the type of cholesteatoma. These findings suggest that identification

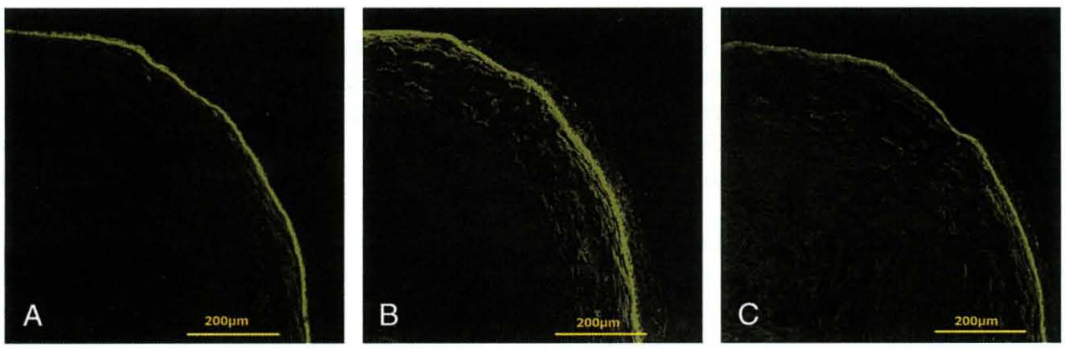

FIG. 2. Reaction time and galectin-7 expression observed by a confocal laser microscope. Invitrogen recommends 60 minutes of reaction time. However, a reaction time of 10 minutes (*C*) was sufficient to identify galectin-7, although the response was a little weak compared to reaction times of 30 (*B*) and 60 (*A*) minutes. Note that cholesteatoma debris was also stained green, assuming a dust particle-like appearance inside the matrix.

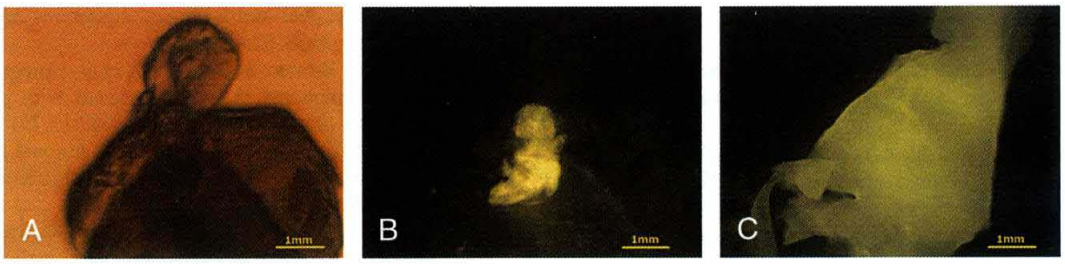

FIG. 3. Stereomicroscopic views of cholesteatoma matrix. The specimens were combined and stained as 1 block without serial sectioning. *A*, Illuminated by natural light. *B*, Illuminated by a blue laser beam. These are photographs of the same specimen. In this case, the specimen was treated with the surrounding granulation and middle ear mucosa. Only cholesteatoma matrix responded well to excitation, corresponding to the presence of galectin-7. *C*, Appearance of the matrix when the whole sample consisted of cholesteatoma.

of cholesteatoma residue is possible by identifying the presence of galectin-7 in the middle ear.

DISCUSSION

Galectin-7 (15.0 kD) is a β-galactoside binding protein that was originally identified in the human epidermis (6) and later found to be expressed in all types of stratified epithelia in the skin, tongue, esophagus, and thymus (7). It plays roles in differentiation and development of the epithelium, notably in epidermis (8), and plays a crucial role in re-epithelialization of epidermal wounds (9). According to Bernerd et al. (10), its expression is rapidly induced after exposure to ultraviolet irradiation, and an increased level of the protein is associated with the apoptotic process in sunburn keratinocytes. Recently, galectin-7 was shown to act as a regulator of apoptosis through c-Jun N-terminal kinase activation and mitochondrial chromosome *c* release. Defects in apoptosis constitute one of the major hallmarks of human cancers, including esophageal (11), hypopharyngeal, and laryngeal carcinomas (12) and malignant lymphoma (13,14). Demers et al. (15) reported that an increased level of galectin-7 expression suggests poor prognosis of malignant lymphoma due to aggressiveness and dissemination of lymphoma cells. Galectin-7 is now considered a useful biomarker to predict prognosis of advanced squamous cell carcinoma of the esophagus (11), hypopharynx (16), and tongue (17); prognosis is predicted to be poor when galectin-7 expression is high in carcinoma cells.

In our previous study, we performed proteomic analysis of human cholesteatoma and found that galectin-7 is a specific protein that is characteristic of cholesteatoma matrix of the middle ear (5). As shown in the present study, this protein was noted in the cholesteatoma matrix but not in the middle ear mucosa. In other words, galectin-7 is expressed only in the multilayered epithelium but not in the monolayered epithelium (9). In healthy individuals, the mucosa in the tympanic cavity is a cuboidal monolayer, whereas the mucosa in other parts is a flat monolayer, except near the orifice of the eustachian tube where the mucosa is multilayered. Because cholesteatoma matrix exists as a multilayer composed of squamous epithelium, identification of galectin-7 in areas other than near the eustachian tube indicates the presence of cholesteatoma matrix. This suggests that identification of galectin-7 using immunofluorescent methods would be a promising way to detect cholesteatoma residue. A similar therapeutic policy was already realized in surgery for malignant glioma, in which the tumor was removed after staining with 5-aminolevulinic acid (18). Furthermore, some investigators have attempted to visualize excision margins of various carcinomas by staining the characteristic protein (19).

The Zenon rabbit immunoglobulin G labeling kit uses a fluorescent dye that produces green light in response to exposure to blue light. The kit is superior in specificity as it uses the immune response to galectin-7. Invitrogen, which is the company that manufactures the kit, recommends 60 minutes to complete the immune reaction. In the present study, we tested 30 and 10 minutes of incubation times. Although image clarity was somewhat lost, recognition of galectin-7 was possible with the shorter staining time. As a result, increases in patient physical stress due to extension of the operation time might be reduced. As Zenon's solution includes sodium azide as an aseptic and preservative agent, its toxicity should be carefully checked before clinical application. We are now conducting an animal experiment concerning the ototoxicity of sodium azide.

CONCLUSION

In the middle ear, galectin-7 was exclusively expressed in cholesteatoma matrix. This indicates that cholesteatoma residue can be detected by identifying this protein at the time of operation. When cholesteatoma residue can be visualized using an immunofluorescent method, complete removal of the matrix becomes easier, thus reducing the incidence of recurrent cholesteatoma.

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