Cell-cycle analysis, growth factors and proliferative activity in middle ear cholesteatoma

Wen-Yuan Chao, M.D.

Department of Otolaryngology, National Cheng Kung University College of Medicine
138 Sheng-Li Road, Tainan 70428, Taiwan
Tel: +886-6-2766697
Fax: +886-6-2377404

Abstract
In order to provide much more understanding into the proliferation and differentiation of the epithelia in cholesteatoma, cell cycle associated molecules were studied by anti-PCNA and anti-Ki67, the two well characterized antibodies. In addition, The experiments included expression, localization, and quantification of EGF, TGF-α; EGF-R in the cholesteatoma. This study demonstrates the presence of TGF-β; EGF and EGF-R in the epithelium and TGF-β; EGF and EGF-R in the stroma of cholesteatoma. As cholesteatoma is a disease with progressive growth of the epithelium, our result showed a high MIB 1/PCNA index means the importance of the inflamed stroma. Taking all the results showing in our study, it may draw a conclusion that TGF-β; EGF, and EGF-R are involved in the formal pathogenesis of cholesteatoma. These results might help us to define the epithelial cells behavior in cholesteatoma clinically.

Keywords—Cholesteatoma, Immunohistochemistry, EGF, FGF-R, TGF-α, PCNA, Ki-67, Proliferation, Differentiation.

Introduction

Cholesteatoma is a common middle ear disease associated with chronic otitis media. It is composed of keratin, epithelial matrix and the subepithelial connective tissue containing granulation tissue and many inflammatory cells [1,2]. Histologically, all the components are benign. Clinically, cholesteatoma continuously grows and invades the surrounding structure in the temporal bone.

Epithelial matrix and subepithelial connective tissue containing numerous inflammatory cells are major components of the middle ear cholesteatoma. The pathogenesis and the factors causing the development and destruction of cholesteatoma have been studying for more than 20 years [1-13]. However, the
mechanism is still not so clear. Our previous study revealed that p53, c-jun/c-fos proteins are cellular components involving in the signal transduction pathway which accounts for the modulating gene expression and protein synthesis [14]. This study applied immunohistochemical staining method to observe the expression, localization and quantification of Epidermal growth factor EGF, the homologous transforming growth factor TGF-α, and epidermal growth factor-receptor EGF-R. Specimens obtained from ear surgery. In addition, cell cycle associated molecules were studied by anti-PCNA and anti-Ki67, the two well characterized antibodies.

Result of this study might provide and account for the contribution of the mitogen, EGF, TGF-α, and EGF-R to the expression of molecular components of signal transduction pathway and the hyperproliferation and differentiation of the epidermal cells in cholesteatoma. So as to define the spatial organization of the cholesteatoma activity. These results might help us to define the epithelial cells behavior in cholesteatoma clinically.

**Materials & Methods**

Forty human middle ear cholesteatoma specimens obtained from patients undergoing middle ear and mastoid surgery were studied. The specimens were fixed in 10% formaldehyde solution and processed for paraffin sections. Serial sections, 6µm in thickness, were cut and divided into 5 groups.

**Immunohistochemical study:**


**Localization for EGF · EGF-R · TGF-α by:**

*Avidin-Biotin-peroxidase-Complex(ABC)-technique*

The following steps were followed —

1. The tissue sections were deparaffinized and hydrated with xylene — alcohol and water
2. Incubated with 0.3 M H₂O₂ in absolute alcohol 20 min
3. Incubated with 3- normal goat serum or 3- normal horse serum
4. Incubated with the primary antibody (1-200)(Rabbit anti-human EGF – Rabbit anti-human EGF-R – Mouse anti-human TGF-x)
5. Incubated with biotin conjugated goat anti-rabbit IgG or biotin conjugated goat anti-mouse IgG
6. Incubated with pre-mixed avidin and biotin-peroxidase at 37° – 45 min
7. 0.01 M H₂O₂ – 0.05 Diaminobenzidine-tetrahydrochloride in PBS
8. Counterstained with hematoxylin

Positive stain were observed as dark brownish color. The omission of the primary antibodies (step 4) were used as negative control. Immunohistochemical demonstration of the color in endothelial cells served as a positive control.

Localization and quantification of Ki-67 and PCNA proteins

Tissue sections were pre-treated with xylene for de-paraffinization and rehydrated. Then were put into 10mM citrate buffer (pH 6.0). Then the slides were heated in a household microwave oven.

1. The tissue slides were incubated overnight with anti-Ki-67 or PCNA at 4°C.
2. The slides were incubated overnight with anti-Ki-67 (1–8) or PCNA (1–20) at 4°C.
3. Incubated with biotin conjugated goat anti-rabbit IgG or biotin conjugated goat anti-mouse IgG
4. Incubated with pre-mixed avidin and biotin-peroxidase at 37° – 45 min
5. 0.01 M H₂O₂ – 0.05 Diaminobenzidine-tetrahydrochloride in PBS
6. Counterstained with hematoxylin

At least 1000 cells were counted in the three – five representative areas at a magnification of 400X. The Ki-67/PCNA score were determined by the quotient of Ki-67/PCNA – positive cells and the total number of cells. Cell nuclei are considered positive if there is any nuclear staining present.

Results

Immunoreactive TGF-β could be shown in all layers of the epidermis of normal meatal skin. There is no difference between staining of the epithelium of cholesteatoma and staining of the epithelium of normal canal skin. Only a few TGF-β positive cells were found in normal external meatal stroma.

Normal middle ear mucosa showed no immunoreactivity for TGF-β. With anti-EGF antibodies no immunoreactivity was found in the epithelial cells of normal
middle ear mucosa and auditory meatal skin, whereas weakly positive immunoactivity was observed in the subepithelial stroma. In normal meatal skin, MIB 1/PCNA-positive cells were mainly found in the stratum basalis. Positive cells were distributed regularly within the basal cell layers. MIB 1/PCNA-positive cells could be observed only infrequently within the normal underlying connective tissue of auditory meatal skin.

As for cholesteatoma tissue, positive immunoreactivity for EGF-R could be demonstrated in basal and suprabasal keratinocytes of epithelium in the majority of the investigated tissue. An increased staining intensity could be demonstrated within adjacent stromal cells of inflamed subepithelial connective tissue. The epithelium of cholesteatoma showed a strongly positive staining for TGF-α throughout the whole epithelium. Enhanced growth factor expression could be demonstrated in endothelial cells, pericytes, fibroblasts, eosinophile leucocytes and macrophages with anti-TGF-α immunostaining. In regions with debris, multinuclear cells were stained positively. Cholesteatoma showed a completely negative staining for EGF in the epithelium, while an intensive staining of mesenchymal cells within inflamed subepithelial stroma could be demonstrated.

Positive immunoreactivity for the proliferation marker could be demonstrated in basal cell layer keratinocytes of epithelium of all 40 cases of cholesteatoma. Some cholesteatomas showed proliferating keratinocytes in the suprabasal cell layers. A heterogeneity of MIB 1/PCNA staining in keratinocytes was detectable in areas of the same case demonstrating an enhanced beside a low mitotic activity. The MIB 1 index of cholesteatoma was 2.3-fold higher, the PCNA-index was 4.4-fold higher as compared to that of external meatal skin revealing a significant statistical difference in the growth pattern of both tissues. The mean number of MIB 1/PCNA-positive keratinocytes increased with the thickness of the epithelium. A higher quality of proliferating keratinocytes was often found within epithelial cones growing towards the underlying stroma.

Discussion

Middle ear cholesteatoma is a common middle ear disease, pathologically benign but clinically destructive on the temporal bone. Cholesteatoma is composed of epithelial matrix and connective tissue including granulation tissue and many inflammatory cells [4, 7].

Clinically, cholesteatoma develops and expands basing on continuing proliferation and differentiation of its epithelial cells. As already known, there’s coexisting with granulation containing numerous inflammatory cells that provide
some factors promoting the proliferation and differentiation of the basal cells of cholesteatoma [2,3,7,8,10,15,16]. The presence of epithelial cells in the middle ear is the characteristics of cholesteatoma. Proliferation and differentiation of the epithelial (basal) cells are the crucial factors in growth of cholesteatoma clinically [1,2,9-11]. Recently, in studying the pathogenesis of cholesteatoma, in addition to the relationship among its composed cells, signal transduction pathway in epithelial cells related to the growth of cholesteatoma is becoming more and more studies in the biological science. Our and others’ previous studies revealed the expression of c-Jun/c-Fos, and p53 in the epithelia and subepithelial connective tissue. This suggests that the signal transduction cascade plays an important role in growth and clinical development of cholesteatoma [17-22].

The demonstration of intensifying immunoreactivity for growth factors like EGF and TGF-β in endothelial cells, pericytes, fibroblasts, macrophages, and eosinophile leukocytes of cholesteatoma underlines a possible stimulatory potency of these cells on growth and differentiation of basal cells of cholesteatoma epithelium. The positive staining within the cytoplasm may represent production and storage of TGF-β, EGF, and EGF-R or ligand-receptor internalisation in keratinocytes and the described stromal cells.

The lack of EGF-R immunoreactivity in the epithelium of middle ear mucosa seems to reveal its incapability to answer the inflammatory stimuli set by TGF-β and EGF. Both factors are present in healing cutaneous wounds [23,24]. They promote epithelial regeneration, keratinocytes migration and angiogenicity, and may lead to an autoinduction of TGF-β production in keratinocytes.

Squamous epithelium is positive for EGF-R staining, in contrast to negative EGF-R staining of middle ear mucosa epithelium. Thus, only the invading epithelium might be the mitotic stimuli of EGF/TGF-β being produced and released by cell of inflamed submucosa. TGF-β is a strong angiogenic factor in vivo [8,15,25,26]. Within the stroma of cholesteatoma, numerous vesicles can be found. Their endothelial cells and pericytes, as well as fibroblasts, eosinophile leukocytes and macrophages were positive for TGF-β immunostaining. The increase of TGF-β immunoreactivity may contribute to the pronounced neovascularization with higher density of microvessels in cholesteatoma, compared to middle ear mucosa and auditory meatal skin. A fact which may lead to an improvement of nutritive supply for the invading cholesteatoma epithelium.

As EGF and TGF-β and their receptor are important regulators of keratinocytes growth, the possibility of autocrine growth of keratinocytes must be discussed [25,26]. In general, the present data describing the distribution of EGF-R in cholesteatoma are not surprising and provide strong evidence to hypothesis that EGF-R is correlated
with proliferation. Both in vivo and in vitro studies suggest that EGF-R is present to an increase amount in proliferative keratinocytes [23]. The positive immunoreactivity for EGF-R mainly in suprabasal layers of epidermis may reflect its hyperproliferative character. An increased turnover of the epithelium induced by inflammation could account for the observation of EGF-R positive keratinocytes in the suprabasal layers.

Cholesteatoma growth is usually accompanied by an inflammatory reaction with eosinophilic infiltration, fibroblast proliferation and neovascularization. These cells as well as endothelial cells, pericytes and macrophages all show positive staining for EGF/TGF-β. The release of these growth factors might induce keratinocyte basal cell proliferation, differentiation and migration. It was suggested that cholesteatoma possess the hyperproliferative character by demonstrating EGF-R in the suprabasal layers[26]. In addition, it also noted that cytokeratin 16, a known marker of hyperproliferation, in all layer of cholesteatoma epithelium [27]. This study showed that positive MIB 1/PCNA immunostaining of keratinocytes in the suprabasal cells layers gives additional evidence of a highly proliferative disease. The wide range of the MIB 1 and PCNA score indicates different proliferative activities within the investigated cholesteatoma specimens. An increased turnover of the epithelium induced by inflammation could account for the observation of MIB 1/PCNA-positive keratinocytes above the basal cell layer [28-33]

**Conclusion**

This study demonstrates the presence of TGF-β and EGF-R in the epithelium and TGF-β, EGF and EGF-R in the stroma of cholesteatoma. As cholesteatoma is a disease with progressive growth of the epithelium, our result showed a high MIB 1/PCNA index means the importance of the inflamed stroma. Taking all the results showing in our study, it may draw a conclusion that TGF-β, EGF, and EGF-R are involved in the formal pathogenesis of cholesteatoma.

**Reference**

4. Chao WY, Huang CC. An immunocytochemical study of cytokeratins expression in


