EPIDERMAL GROWTH FACTOR EXPRESSION IN MIDDLE EAR CHOLESTEATOMA

Hung-Pin Chi, Kuen-Yao Ho, Chee-Yin Chai, Chih-Feng Ta, Ling-Feng Wang, Ka-Wo Lee, Wen-Rei Kuo, Shiu-Chuan Wu, and Shin-Meng Tsai
Departments of Otolaryngology, Pathology, Surgery, and Public Health, Kaohsiung Medical University, Kaohsiung, Taiwan.

Middle ear cholesteatoma is destructive to auditory ossicles and temporal bone, and treatment usually requires surgical removal of all epithelial content. Epidermal growth factor (EGF) can stimulate the growth and differentiation of a variety of mammalian cells, including epithelial cells. Our study used the avidin-biotin complex technique to evaluate the expression of EGF in 40 cases of middle ear cholesteatoma (active cholesteatoma, 31 cases; inactive cholesteatoma, 9 cases) and 34 normal postauricular skin samples. In middle ear cholesteatoma, EGF was expressed in squamous epithelium in 21 cases (53%), fibroblasts in two cases (5%), and cholesteatoma endothelium in two cases (5%). In normal postauricular skin, EGF was expressed in squamous epithelium in 14 samples (41%), fibroblasts in one sample (3%), and endothelium in none. No statistical difference in EGF expression was found between cholesteatoma and normal postauricular skin samples. These results show that the distribution of EGF in middle ear cholesteatoma is not deranged and that the progression of cholesteatoma might be induced by the release of factors from the cholesteatoma matrix via autocrine stimulation, or by inflammatory cells of the subepithelial tissue through paracrine stimulation, or in both of these ways.

Key Words: epidermal growth factor, middle ear cholesteatoma

Cholesteatoma is a destructive lesion of the middle ear or mastoid process that produces complications through erosion of the temporal bone with involvement of the contained neural and vascular structures and adjacent central nervous system structures. Histologically, cholesteatoma is composed of an accumulation of desquamated keratin arising from the squamous epithelium [1]. Recent research has attributed an important role in keratinocyte proliferation to numerous growth factors and cytokines, and one of these is epidermal growth factor (EGF) [2]. EGF, a single-chain polypeptide consisting of 53 amino acids, can stimulate the growth and differentiation of a variety of mammalian cells, including epithelial cells. The level of EGF (in mammalian cells, including epithelial cells) is related to the state of differentiation and the ability of keratinocytes to differentiate [3]. Its expression in keratinocytes, which depends on the state of differentiation, may be considered a marker indicating the state of proliferation and terminal differentiation [4]. Using immunohistochemistry, we attempted to elucidate the site and degree of EGF localization in different types of cells involved in middle ear cholesteatoma. Immunohistochemical staining indicates the sites where EGF may be synthesized. We aimed to identify the expression of EGF in examined cells and the role EGF plays in the growth of cholesteatoma.

MATERIALS AND METHODS
Cholesteatoma specimens were obtained from 40 patients (mean age, 38 years; range, 19–67 years) who underwent
middle ear surgery at the Department of Otorhinolaryngology, Kaohsiung Medical University Hospital, in 2000 to 2002. Specimens from 31 patients with a thicker matrix (accumulation of keratin debris due to active proliferation and differentiation of keratinocytes) were labeled active cholesteatoma. Specimens from the remaining nine cases with less debris and granulation tissue or thin matrix were labeled inactive cholesteatoma.

For the control, normal postauricular skin specimens were obtained from the 40 patients as they underwent middle ear surgery. If any inflammation or cholesteatoma cells were suspected to be present, the specimens were excluded; 34 patients were finally included (mean age, 43 years; range, 19–51 years).

**Immunohistochemical staining**

Immediately after surgery, specimens were fixed in 10% buffered formalin solution overnight, dehydrated, embedded in paraffin, and sliced into 4 μm sections. After deparaffinization and washing, endogenous peroxidase was inhibited using a methanolic solution of 0.3% hydrogen peroxide and then blocked with 10% normal horse serum and 1% bovine serum albumin for 30 minutes. After a brief rinse, sections were incubated with the EGF receptor (EGF-R) monoclonal antibody (1:20; BioGenex Laboratories Inc, San Ramon, CA, USA) at room temperature for 120 minutes. They were then washed in phosphate-buffered saline, incubated for 30 minutes with biotinylated anti-mouse immunoglobulin G (IgG), and treated with avidin-biotin complex for 30 minutes; 3,3'-diaminobenzidine was then applied as a chromogen. Sections were visualized after counterstaining with Mayer’s hematoxylin. EGF labeling was considered positive if one EGF-positive cell was present. Omission of the primary antibody was used as a negative control and EGF expression in breast cancer samples was used as a positive control (brownish color and gloss strength [5]. Differences between the distribution patterns in the squamous epithelium, fibroblasts, and endothelial cells in the two groups (cholesteatoma and normal postauricular skin) were tested using the Chi-squared test.

**RESULTS**

**Middle ear cholesteatoma**

EGF expression in the cytoplasm of cholesteatoma epithelium (Figure 1) was the same as that in breast cancer (Figure 2). In the 31 cases of active cholesteatoma, EGF staining was strongly positive or positive in 17 cases (55%) in the squamous epithelium, in two cases (6%) in fibroblasts, and in two cases (6%) in the endothelium (Table 1). Four of the nine cases (44%) of inactive cholesteatoma had strongly positive or positive EGF staining in the squamous epithelium (Table 1). Active cholesteatoma showed more EGF immuno-reactivity than inactive cholesteatoma. EGF immuno-reactivity was greatest in the squamous epithelium and decreased in the fibroblasts and endothelium.

**Postauricular skin**

In the 34 normal postauricular skin samples, EGF staining was positive in 14 cases (41%) in the squamous epithelium, in one case (3%) in fibroblasts, and in no cases in the endothelium (Table 2). Cholesteatomas had greater EGF expression than normal postauricular skin in the squamous epithelium, but this did not reach statistical significance.
DISCUSSION

Middle ear cholesteatoma is a pathologic condition that develops as a result of the extension or depression of the flaccida or tensa of the tympanic membrane. Most cholesteatomas concomitantly have an inflammatory reaction in keratinocytes. Inflammatory cells may actively participate in the aberrant functioning of the cholesteatoma epithelium by releasing various cytokines. These are then involved in hypervascularization, a migratory and invasive epithelium, and hyperproliferation [1,6]. The growth of cholesteatoma seems not to cease and the cholesteatoma may recur many years postoperatively unless it is completely eradicated. The clinical characteristics of cholesteatoma indicate that it may have a hyperproliferative ability and that progression may be induced by the release of factors from the cholesteatoma matrix via autocrine stimulation, by inflammatory cells of the subepithelial connective tissue via paracrine stimulation, or in both ways [7]. Different growth factors and cytokines have been identified as being involved in the pathogenesis of cholesteatoma. EGF, transforming growth factor α (TGF-α), and interleukin 1 (IL-1) seem to be responsible for the increased proliferation rate of keratinocytes [3].

EGF is a single-chain polypeptide made up of 53 amino acids. It has been structurally well characterized in both animals and humans. Its numerous biologic effects include stimulation of extracellular compounds transported to epithelial cells, activation of glycolysis, initiation of DNA synthesis, activation of RNA and protein synthesis, and increased synthesis of extracellular macromolecules. EGF can induce the proliferation of epithelial and endothelial cells, fibroblasts and keratinocytes [4,8]. In this study, we used immunohistochemical staining to identify the distribution of EGF in several cell types in cholesteatoma and evaluated the role and mechanism of action of EGF in cholesteatoma.

More EGF-positive staining was found in the squamous epithelium in active cholesteatomas than inactive cholesteatomas, which showed that active cholesteatoma epithelium proliferated faster than inactive cholesteatoma epithelium. In postauricular skin, there was more EGF staining in the squamous epithelium than in other tissues. The orderly pattern of EGF staining was not deranged in cholesteatoma epithelium, which was similar to that in normal postauricular skin. This may reflect the high proportion of inactive cholesteatomas and apoptosis in our specimens, which led to no significant difference in EGF expression in cholesteatoma and normal skin tissue.

Angiogenesis is particularly important in pathologic processes, including inflammation. Because proliferating tissues such as middle ear cholesteatoma require an enhanced blood supply, angiogenesis appears to be a prerequisite for the expansion of the cholesteatoma matrix.
Angiogenesis enables and supports the sustained migration of keratinocytes into the middle ear cavity [2]. Previously, TGF-α and EGF were thought to be potent angiogenic factors that stimulated endothelial cells. Sudhoff et al examined angiogenesis in middle ear cholesteatoma and found overexpression of both EGF and EGF-R, raising the possibility that EGF stimulates endothelial cells and so induces angiogenesis [7]. In this study, EGF staining of endothelium was more common in active than in inactive cholesteatoma, but there was no significant difference between inactive cholesteatoma and normal postauricular skin. Because monocytes, macrophages, and infiltrating leukocytes are known to produce cytokines such as EGF and TGF-α, angiogenesis might be induced by inflammatory cells. These processes may be complicated by many growth factors and may be closely related to the degree of inflammation. Our study suggests that EGF is also a product of endothelial cells and so is a candidate for involvement in the development of cholesteatoma not only through paracrine regulation but also via autocrine regulation.

Hildmann and Sudhoff reported that EGF expression is related to the degree of inflammation within the perimatrix [9]. There was no significant difference in EGF distribution between active and inactive cholesteatoma and postauricular skin in our study. Palacios et al reported that the stimulation effect of EGF is dose-dependent [10]. At lower concentrations, EGF has a stimulatory effect on mucosal epithelium. However, at the highest concentration, it appears to inhibit cellular growth. Increased EGF expression in middle ear cholesteatoma has been described for epithelial cells [11, 12], and high EGF levels have been demonstrated in cholesteatoma debris [13]. In the human ear, active cholesteatomas have greater immunoreactivity with EGF in their epidermal layers than inactive cholesteatomas [14]. Bujia et al found that basal cells of the epidermis and human cholesteatoma contain high concentrations of EGF and cell receptors [3]. Fibroblasts of cholesteatoma are more aggressive than fibroblasts of ear canal skin, which seems to be due to EGF. EGF activates tyrosine protein kinase in the receptor, which induces mitotic activity. Yetiser et al showed that higher levels of cytokines in patients with cholesteatoma confirm that the destructive behavior of cholesteatoma is likely to be mediated by cytokines and EGF and is the result of keratinocyte activity [15].

How does the epithelium grow into the middle ear? There are many theories, including the metaplasia hypothesis, the retraction theory and direct ingrowth from eardrum perforation. The squamous epithelium that migrates into the middle ear cavity uses granulation tissue and the organized effusion as a bridge, resulting in migration and cholesteatoma formation [16]. Besides the proliferation effect, EGF also has effects in supporting both cell cycle progression and keratinocyte survival. EGF may help keratinocytes to survive and proliferate in different conditions, such as middle ear mucosa or even bony substrate. Kojima et al used in situ hybridization to show that EGF is produced by the basal cells of the epithelial layer of cholesteatoma [11]. The mechanism of epithelial basal cell proliferation is through an autocrine system via EGF and EGF-R.

Whether the cholesteatoma epithelium has more hyperproliferative ability than normal skin is controversial, although a previous study drew this conclusion [17]. Sudhoff et al reported EGF immunoreactivity in neither cholesteatoma epithelium nor in middle ear mucosa residues [18]. Zymographic analysis of culture supernatants from 10 cholesteatoma and four meatus skin samples showed no statistically significant differences between controls and cholesteatoma samples [19].

Previous studies used immunochemistry to investigate proliferating cell nuclear antigen in cholesteatoma and evaluated its proliferative activity. Their results indicate that cholesteatoma has a similar proliferative activity to normal postauricular skin, and that cholesteatoma itself is not a tumor despite its clinical behavior, which is similar to neoplastic cells [14,19,20]. Furthermore, Albino et al found neither obvious molecular nor cellular differences among the various types of cholesteatoma, and that cholesteatoma epithelium behaved more like a wound-healing process than a neoplasia [21]. Kojima et al found EGF in only 20% of normal epidermal epithelium and 50% of cholesteatoma epithelium [11], which suggests that other growth factor(s) may also be needed to regulate growth of cholesteatoma epithelium.

Although more EGF positive staining was observed in the epithelium of active cholesteatoma, we did not observe any significant difference between cholesteatoma and postauricular skin in this respect. Results from the current study suggest that EGF might influence the development of cholesteatoma (including active and inactive types), but it may not be the only factor. Other factors such as the environment of the cholesteatoma, other growth factors, and inflammatory status may also play a role [4]. This observation seems to support the hypothesis that cholesteatoma epithelium has similar proliferative activity to postauricular skin tissue or benign tumor [22]. Bujia et al reported that there were close similarities between psoriasis and middle ear cholesteatoma concerning hyperproliferative behavior [3]. EGF from squamous epithelium, endothelium, and
fibroblasts may play an important role as one of the biochemical factors by binding to EGF receptors through an autocrine or paracrine mechanism. It is hoped that further studies will provide better understanding of this interaction.

REFERENCES