Angiogenesis and Angiogenic Growth Factors in Middle Ear Cholesteatoma

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Hypothesis: This study aimed to analyze the localization and distribution of vessels and of these angiogenic growth factors: basic fibroblast growth factor (FGF-2), transforming growth factor-α (TGF-α), transforming growth factor-β1 (TGF-β1), and vascular endothelial growth factor (VEGF) in middle ear cholesteatoma in comparison with normal middle ear mucosa and auditory meatal skin.

Background: Angiogenesis is particularly important in many normal and pathologic processes, including wound healing and inflammation. Because proliferating tissues require an enhanced blood supply, angiogenesis appears to be a prerequisite for the expansion of cholesteatoma.

Methods: The expression of FGF-2, TGF-α, TGF-β1, and VEGF was studied by immunohistochemistry. The amount of vessels (collagen type IV staining) was determined by an automatic imaging analyzing system.

Results: The results showed an altered expression and distribution of VEGF, FGF-2, TGF-α, and TGF-β1 in cholesteatoma in relation to middle ear mucosa and auditory meatal skin. The results were consistent with rapidly growing, activated keratinocytes and stromal cells. Vascularization within the perimatrix of cholesteatoma showed a 4.3-fold increase compared with middle ear mucosa and a twofold increase compared with ear canal skin. An increase of 3.2- to 4-fold in the number of vessels was observed. A close relationship was seen between the density of capillaries, degree of inflammation, and expression of the angiogenic factors investigated, and an increased number of microvessels in cholesteatoma tissue.

Conclusions: Angiogenesis enables and supports the sustained migration of keratinocytes into the middle ear cavity. Therefore, it is a pivotal factor in the destructive behavior of middle ear cholesteatoma. Key Words: Cholesteatoma—Middle ear—Angiogenesis—Growth factors—Collagen type IV—Fibroblast growth factor-2—Transforming growth factor-α—Transforming growth factor-β1—Vascular endothelial growth factor—Immunohistochemistry.


Growth factors are proteins that control the growth and differentiation of cells in multicellular organisms. Unlike hormones, they are synthesized locally at their site of action. These proteins are called growth factors because one of their many actions is to strongly influence cell growth and division. Because proliferating tissues like middle ear cholesteatoma have an enhanced proliferative activity, the release of growth factors appears to be a requirement for the expansion of cholesteatoma matrix within the middle ear cavity (1,2). Several authors have searched for substances in cholesteatoma tissue that could be responsible for its considerably altered growth properties (3–6). A role for several cytokines and growth factors in the development and proliferation of cholesteatoma tissue and the resultant destructive behavior has been proposed (3–6). The progression of cholesteatoma might be induced by the release of factors from the matrix of cholesteatoma in an autocrine stimulation, by inflammatory cells of the subepithelial connective tissue in a paracrine stimulation, or in both ways.

Angiogenesis is the formation of new blood vessels from established microcirculation (7). It is particularly important in many normal and pathologic processes, including wound healing and inflammation (8,9). Because proliferating tissues like middle ear cholesteatoma require an enhanced blood supply, angiogenesis appears to be a prerequisite for the expansion of cholesteatoma ma-
Because the process of angiogenesis occurs in response to specific growth factors and cytokines, we were interested in characterizing these factors in cholesteatoma tissue (7,9). To date, several factors have been identified that affect endothelial cells directly or indirectly, like fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), interleukin-1 (IL-1), and interleukin-6 (IL-6) (7,9–11).

Fibroblast growth factor-2 is an 154-amino acid polypeptide with a molecular weight of about 18 kDa. It is mitogenic in vitro for fibroblasts, endothelial cells, and keratinocytes (12). In vitro, FGF-2 is also an important morphogen for endothelial cells, and it enhances the production of extracellular matrix by these cells (12). In the middle ear, FGF-2 is produced in cells surrounding blood vessels and close to the basement membrane during otitis media (13,14). These properties are consistent with a role of FGF-2 in angiogenesis. Transforming growth factor-β1 is a 25-kDa homodimer composed of two 12.5-kDa subunits joined by disulfide bonds (11). It affects cellular proliferation, cellular differentiation, and gene expression of all major cell types in wound healing (11). This growth factor regulates many of the processes in both tissue repair and disease, including angiogenesis, chemotaxis, and fibroblast proliferation, as well as synthesis and degradation of matrix proteins like collagen and fibronecnotin (15). Also, TGF-β1 inhibits keratinocyte proliferation in submersed cell cultures (15). Vascular endothelial growth factor, a dimeric glycoprotein of 34 to 45 kDa, is a potent multifunctional cytokine (16). It shares low but significant homology with platelet-derived growth factor, and it contributes to angiogenesis by both direct and indirect mechanisms (16,17). It stimulates the endothelial cell lining near microvessels to proliferate, to migrate, and to alter their pattern of gene expression (17). Additionally, VEGF renders these same microvascular endothelial cells hyperpermeable so that they spill plasma proteins into the extravascular space, leading to the clotting of extravasated fibrinogen with deposition of a fibrin gel (17).

In many tissue systems, the differentiation and proliferation of cells is known to be regulated by growth factors, like VEGF, FGF-2, TGF-β1, TGF-α, and EGF. Thus, it seems reasonable to assume that middle ear cholesteatoma growth is similarly controlled. Consequently, we investigated the expression and localization of some pivotal angiogenic growth factors in cholesteatoma in comparison with normal auditory meatal skin to identify some of the growth factors involved in the pathogenesis of middle ear cholesteatoma.

**MATERIALS AND METHODS**

All tissue samples were obtained from patients requiring middle ear surgery. Twenty-two cholesteatomata, 8 normal auditory canal wall skin, and 5 normal middle ear mucosa specimens were immediately fixed in formalin and embedded in paraffin. All specimens were examined histopathologically with routine hematoxylin and eosin staining.

The following antibodies were used for immunohistochemistry: FGF-2 (monoclonal), VEGF (monoclonal), TGF-β1 (polyclonal) (all from R&D Systems, Wiesbaden, Germany, diluted 1:40), TGF-α (monoclonal) (Dianova, Hamburg, Germany, diluted 1:300), and collagen type IV ( monoclonal) (DAKO, Hamburg, Germany, diluted 1:40). Immunohistochemical staining was performed by using the avidin-biotin–complex technique (ABC) according to Hsu et al. (18). Serial 4-μm paraffin sections were placed on poly-L-lysine–coated slides (10% poly-L-lysine, Sigma, Deisenhofen, Germany), deparaffinized, hydrated, and dried overnight at 37°C. Endogenous peroxidase activity was eliminated with 3% H2O2 at room temperature for 10 minutes and nonspecific protein binding by incubation with 10% normal goat serum for polyclonal primary antibody or 10% normal horse serum for monoclonal antibody at room temperature for 20 minutes. The slides were then incubated with the primary antibody at 4°C overnight. After washing with phosphate-buffered saline, the sections were incubated for 30 minutes with a secondary biotinylated antibody (Vectastain Kit, Camon Laboratories, Wiesbaden, Germany), followed by incubation with a complex of avidin and biotinylated peroxidase over 30 minutes at room temperature. Color development was obtained with diaminobenzidine (Camon Laboratories, Wiesbaden, Germany), yielding a brown precipitate. Sections were counterstained with Meyer’s hematoxylin and mounted with synthetic mounting medium (Histogel, Camon Laboratories). Preimmune sera and the omission of the primary antibodies were used as negative controls. Tissue specimens were examined and photographed with a Zeiss (Jena, Germany) light microscope.

The intensity of immunohistochemical staining was considered to be negative (−) (no reaction), weakly positive (+) (<5% positive cells), definitely positive (+++) (5%–75% positive cells), or strongly positive (++++) (>75% positive cells). The degree of inflammation of the cholesteatoma perimatrix was evaluated by two different observers and classified into Grades I to III. Morphologic analysis and microvessel density were determined by an automatic analyzing system (KS300, Kontron Elektronik, Köln, Germany). Collagen type IV–positive stained blood vessels within 1 mm underneath the subepithelial basement membrane were quantified irrespective of their morphology. As morphologic parameters, vessels per area (number per millimeter2) and relative share of the total vessel area in relation to the total area of connective tissue (percent) were analyzed. The Student-Newman-Keuls test was used for statistical evaluation (significance level 0.05).

**RESULTS**

**Normal middle ear mucosa and external auditory meatal skin**

The mean number of microvessels counted within the subepithelial connective tissue of normal middle ear mucosa was 5.3 ± 1.2 vessels/mm2. The mean relative proportion of the total vessel area to the total area of connective tissue was 2.5 ± 0.8% (Figs. 1, 2; Table 1). The subepithelial connective tissue of normal auditory meatal skin contained 6.5 ± 2.2 vessels/mm2 with a relative area proportion of 5.5 ± 2.0% (Figs. 1, 2; Table 1). By immunohistochemistry, we could not detect any reactivity for TGF-β in middle ear mucosa or external auditory...
meatal skin. The subepithelial and vascular basement membranes showed positive immunoreactivity for VEGF and FGF-2 (Fig. 3 A). Normal auditory meatal skin labeled with anti-VEGF antibody showed a low to moderate labeling throughout the epidermis except for the stratum corneum. Transforming growth factor-β1 was demonstrated in keratinocytes of the squamous epithelium as well as in the endothelial cells (not shown) (Table 1). Consistent results were obtained for the immunohistochemical expression patterns of the investigated factors in normal mucosa and ear canal skin.

**Middle ear cholesteatoma**

The mean number of microvessels found within the perimatrix of all cholesteatoma was determined to be $21.1 \pm 11.7$ vessels/mm$^2$ ($n = 22$). The mean number of vessels increased with the degree of inflammation within the perimatrix (Grades I–III) from $9.0 \pm 3.5$ vessels/mm$^2$ (Grade I) to $19.2 \pm 3.6$ vessels/mm$^2$ (Grade II) (Figs. 1, 2) and $31.7 \pm 9.4$ vessels/mm$^2$ (Grade III) (Figs. 1, 2) ($p < 0.05$) (Table 1). The mean proportion of the total vessel area to the total area of the perimatrix was $10.8 \pm 2.9\%$ for all 22 cholesteatoma samples, showing an increase from $7.7 \pm 2.3\%$ (Grade I) to $11.0 \pm 1.7\%$ (Grade II) and $13.2 \pm 1.4\%$ (Grade III) (Figs. 1, 2, Table 1) ($p < 0.05$).

A strongly positive immunoreactivity for TGF-α could also be detected in basal and suprabasal keratinocytes of the epithelium of cholesteatoma. An increased staining intensity was found within adjacent stromal cells of inflamed subepithelial connective tissue and endothelial cells (not shown). Definite differences also became obvious between cholesteatoma perimatrix and the subepithelial connective tissue of normal middle ear mucosa and auditory skin concerning TGF-β1, FGF-2, and VEGF immunoreactivity. Numerous infiltrating cells within the perimatrix expressed high amounts of TGF-β1, whereas no immunoreactivity was observed within the matrix (Fig. 4, Table 1). The immunostaining of subepithelial and vascular basement membranes for FGF-2 was apparently diminished and revealed focal disruptions or dissolved in cholesteatoma tissue. Numerous cells of the immune cell infiltrate, presumably monocytes, macrophages, and mast cells, were stained positive with anti-FGF-2 and anti-VEGF antibodies within the cytoplasm (Figs. 3 B and 5). Additionally, a higher number of basal keratinocytes in cholesteatoma tissue stained positive for VEGF than in ear canal skin (Fig. 5, Table 1). The immunohistochemical expression patterns of the different growth factors varied within the cholesteatoma samples depending on the local inflammatory infiltrate.

**DISCUSSION**

The pathogenesis of middle ear cholesteatoma comprises a complex and dynamic series of events in which cellular and extracellular components show an altered table

**TABLE 1.** Comparative synopsis on immunohistochemical and morphometric findings in normal mucosa, ear canal skin, and cholesteatoma with increasing degrees of inflammation (I–III)

<table>
<thead>
<tr>
<th>Degree of inflammation</th>
<th>FGF-2</th>
<th>VEGF</th>
<th>TGF-β1</th>
<th>TGF-α</th>
<th>Vascularization (%)</th>
<th>Vascularization (vessels/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosa</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>2.5 ± 0.8</td>
<td>5.3 ± 1.2</td>
</tr>
<tr>
<td>Ear canal skin</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5.5 ± 2.0</td>
<td>6.5 ± 2.2</td>
</tr>
<tr>
<td>Cholesteatoma</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>11.0 ± 1.7</td>
<td>19.2 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>13.2 ± 1.4</td>
<td>31.7 ± 9.4</td>
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</tbody>
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The American Journal of Otology, Vol. 21, No. 6, 2000
biologic behavior (3–6,19,20). These processes are mediated by many growth factors and cytokines that have been only partially identified.

During the course of cholesteatoma development, we observed chronic and acute inflammatory reactions within the perimatrix, revealing several parallels to wound repair (20,21). With increasing degrees of inflammation, we could demonstrate an enhanced vascularization within the perimatrix (Table 1). Because monocytes, macrophages, and infiltrating leukocytes are known to produce cytokines, like FGF-2, VEGF, EGF, TGF-β, IL-1, and IL-6, angiogenesis might be induced by inflammatory cells. On the other hand, it could be shown that keratinocytes of cholesteatoma are able to release some angiogenic factors and, as well, could induce or contribute to the observed angiogenesis. The predominance of mainly small vessels with small microvessel diameters gives further evidence to the formation of new blood vessels from established microcirculation in middle ear cholesteatoma.

When normally quiescent endothelial cells that line venules are stimulated, they will degrade their basement membrane and adjacent extracellular matrix, migrate directionally, divide, and organize into new functioning vessels invested by newly formed basement membrane (10,12). A loss of the basement membrane components collagen type IV and laminin in middle ear cholesteatoma has already been described by us (21). As a component of the basement membrane bound to proteoglycans,
FGF-2 is one of the most potent angiogenic factors. Degradation of basement membrane by infiltrating leukocytes will result in the liberation of matrix-binding factors like FGF-2, a mechanism that could substantially contribute to the enhanced vascularization of cholesteatoma perimatrix (12–14, 22). Additionally, the infiltrating leukocytes themselves are another source of the growth factors investigated. Comparable findings have been described for the healing of traumatic tympanic membrane perforations. In normal tympanic membrane, FGF-2 was not detected, but it was expressed 3 days after a traumatic perforation, mainly in the perforated area in pericytes and in polynuclear cells. Similarly to our findings in cholesteatoma tissue, healing in tympanic membrane acute perforation repair is also associated with fibroblast growth factor upregulation after damage (23).

Vascular endothelial growth factor is a multifunctional cytokine that contributes to angiogenesis by both direct and indirect mechanisms (6). It stimulates the endothelial cells lining nearby microvessels to proliferate, to migrate, and to alter their pattern of gene expression; also, it renders these same microvascular endothelial cells hyperpermeable so that they spill plasma proteins into the extravascular space, leading to the clotting of extravascular fibrinogen with deposition of a fibrin gel (17). Extravascular fibrin serves as a provisional matrix that favors and supports the ingrowth of new blood vessels and other mesenchymal cells that generate a vascularized perimatrix. The expression of VEGF in endothelial cells most likely represents uptake rather than synthesis, because there is little evidence of VEGF production by endothelial cells. Moreover, activated immunocompetent cells and fibroblasts, which were frequently labeled for VEGF, as reported for other diseases, may exert paracrine influences in concert with epithelial cells and contribute to angiogenesis in middle ear cholesteatoma (12–14).

Previously, TGF-α and EGF have been described in cholesteatoma tissue with respect to keratinocyte proliferation. Additionally, both factors are potent angiogenic factors but do not increase microvascular permeability (24). However, TGF-α strikingly upregulates VEGF expression in cultured keratinocytes and is thought to be responsible, at least in part, for the overexpression of VEGF in hyperproliferative skin diseases (24, 25). Moreover, overexpression of TGF-α and EGF, along with that of the EGF receptor with which it interacts, raises the possibility that TGF-α and EGF act to stimulate endothelial cells and in this manner induce angiogenesis. In many instances TGF-β1, which is initially expressed as an inactive molecule, is inhibitory to cell proliferation and inflammation (26). The concomitant presence of growth inhibitory factors (TGF-β1) as well as growth promoting factors (VEGF) and cell maintenance/survival factors (FGF-2) in cholesteatoma can be interpreted as representing a system of upregulated controlled growth that is near steady state, rather than uncontrolled growth.

In conclusion, our results show an increased expression of VEGF, FGF-2, TGF-β1, and TGF-α in cholesteatoma, which may initiate and promote angiogenesis. Our findings suggest that angiogenesis is inaugurated in middle ear cholesteatoma and is closely related to the degree of inflammation and release of the angiogenic growth factors. Endothelial cell proliferation could be induced by the release of angiogenic factors from cholesteatoma matrix or by inflammatory cells of the subepithelial connective tissue. Additionally, the dual growth factor phenomenon of autocrine stimulation of cell proliferation and paracrine stimulation of the surrounding cells may play an essential role. Angiogenesis enables and supports the sustained migration of keratinocytes into the middle ear cavity. Therefore, it is a pivotal factor for the destructive behavior of this middle ear lesion. Further studies on new treatment strategies by inhibiting FGF or VEGF may reveal whether this therapeutic principle could be suitable for a therapy of cholesteatoma concomitant with middle ear surgery.

REFERENCES


