A Study of Inflammatory Mediators in the Human Tympanosclerotic Middle Ear

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Objective: To analyze immunocompetent cells as well as 2 factors involved in inflammation and also thought to be involved in bone remodeling—interleukin 6 (IL-6) and inducible nitric oxide synthase in the human middle ear, including the tympanic membrane.

Design: Biopsy specimens were obtained from the human middle ear and tympanic membrane during surgery. Using an immunohistochemical technique, the expression of macrophages, T cells, B cells, IL-6, and inducible nitric oxide synthase were analyzed.

Materials: Nine biopsy specimens from tympanic membranes in children having a transtympanic ventilation tube inserted as a treatment for secretory otitis media and 11 biopsy specimens from tympanosclerotic plaques from patients with chronic otitis media and tympanosclerosis.

Results: More positively stained specimens showing macrophages, B cells, and IL-6 were seen in the biopsy specimens from children with secretory otitis media compared with the biopsy specimens from patients with chronic otitis media and tympanosclerosis. The biopsy specimens from patients with chronic otitis media and tympanosclerosis more often showed positive stainings for inducible nitric oxide synthase than the biopsy specimens from children with secretory otitis media. The presence of IL-6 and inducible nitric oxide synthase was shown by staining to be mostly in the surface cells, while macrophages and B cells were stained deeper in the tissues, in connective tissue, or around sclerotic lesions.

Conclusions: The 2 patient groups differed in antigen presentation so that macrophages, B cells, and IL-6 were labeled more frequently in patients with secretory otitis media, that is, an early phase of the disease. Inducible nitric oxide synthase was seen more frequently in the patients with already established tympanosclerosis in a later phase of the disease.

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Tympanosclerosis (TS) is a condition that occurs in the middle ear, including the tympanic membrane (TM), and presents itself as calcification of connective tissue. The process is most often seen in the TM but may also involve other sites in the middle ear.

Tympanosclerosis is seen as white chalky patches in the middle ear or in the TM. It is, in the initial stages, seen as cheeselike masses of sclerotic material and in the later stages as a harder, more bone-like material. Tympanosclerosis has been well described by many investigators. The TS plaques are located in the lamina propria of the TM. The microstructure, as seen under the electron microscope, is composed of an irregular 3-dimensional collagen lattice, enclosing spherical mineralized aggregates that are masses of calcium phosphate. The calcification process resembles that occurring in other diseases such as arteriosclerosis. The patients suffering from TS usually have a history of either acute or chronic otitis media. When the TM is solely engaged, the disease is usually defined as myringosclerosis (MS). The TM also involves the most common localization of the process. Myringosclerosis often occurs in patients who underwent tympanoplasty and had ventilation tubes inserted. Tympanosclerosis can occur after a mild inflammatory process as well as after severe repeated bouts of inflammatory disease. The incidence of TS in previously analyzed materials from patients with otitis media varies between 20% and 43%. Some patients develop hearing loss when the middle ear ossicles are more or less fixed as a result of the TS process. The inner ear can also be affected in severe cases in which the otic capsule is involved. This may lead to sensorineural hearing loss and even deafness in rare cases. Depending on the

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Patients, Materials, and Methods

Patients

We studied 2 patient groups from whom biopsy specimens were obtained. Nine children (4 girls and 5 boys) with SOM who underwent the placement of middle ear ventilating tubes were randomly selected. The children ranged in age between 2 and 9 years (Table 1). All patients showed intact TMs with the appearance of clear effusion behind them. No signs of purulent otitis media was present. Despite the appearance, 1 middle ear was found to be aerated. The surgery was performed with the indication of a hearing disability. A special instrument was used to obtain a biopsy specimen, creating a small hole in the TM, where the ventilation tube was placed. These specimens are hereafter referred to as the SOM biopsy specimens.

Eleven specimens from defined TS plaques in the TM or the middle ear from patients who underwent tympanoplasty were removed during surgery. Ten of the patients were females and 1 was male. Age ranged from 8 to 55 years (Table 2). All patients had dry TM perforations prior to surgery. There was no history of otorrhea for several months in these patients, except for 1 case in which ongoing otorrhea was present at the time of surgery. All patients had some kind of conductive hearing loss. The biopsy specimens were collected from patients being operated on under general or local anesthesia. These specimens hereafter are referred to as the TS biopsy specimens. The project was approved by the Swedish Ethical Board (No. 92:40). All patients had been informed and gave their consent. The information was verbal and written. If the patient was younger than 18 years, the parents were informed and gave their consent.

Materials and Methods

All biopsy specimens were immediately fixed in 4% paraformaldehyde for 1 hour, then embedded in paraffin. They were subsequently serially sectioned in 4-µm sections and put on glass slides. Thereafter, they were deparaffinized. Blocking of endogenous peroxidase activity was performed by using 0.3% hydrogen peroxide. The sections were preincubated with normal serum from the same animal in which the secondary antibody was produced. The slides were then exposed to the primary antibodies MAC 387, CD3, CD20, iNOS, and IL-6 (1H-183) (Table 3).

The biopsy specimens were incubated in darkness overnight. After rinsing, the secondary antibody was applied. When the primary antibody was of monoclonal, biotinylated immunoglobulin-antimouse-IgG (Vectastain; Vector Laboratories, Burlingame, Calif) was used. For polyclonal antibodies, biotinylated anti–rabbit IgG was used (Scandinavian Diagnostic Services, Copenhagen, Denmark). Avidin–biotin complexes (Vectastain) were added and the specimens were incubated with 3-amino-9-ethylcarbazole, dimethylformamide, and 30% hydrogen peroxide, pH 5.0. The slides were kept in darkness for 4 minutes, then rinsed with tap water. The sections were counterstained with Mayer hemalum. Specimens in which the primary antibody had been replaced by normal serum and phosphate-buffered solutions were used as negative controls. In staining procedures including MAC 387, CD3, CD20, and iNOS, human tonsil tissue, known to contain immunocompetent cells, was used as both a positive and negative (Figure 1) control. When staining for IL-6, rat lymph nodes were used as negative and positive controls. The specimens were analyzed and photographed using a light microscope (Axioplan; Carl Zeiss Ltd, Oberkochen, Germany). Since the biopsy specimens varied in size and shape, it was difficult to compare the number of positive cells in each section; therefore, the specimen was considered either positive or negative for antibody staining. Data from the positive specimens were analyzed using the Mann-Whitney test. The results are summarized in Table 1 and Table 2. Statistical significance was set at P<.05. Table 3 lists the different antibodies used in detail.

Localization, there are different implications for the choice of treatment modalities (eg, surgical procedures). When performing surgery, there is always a risk of creating iatrogenic sensorineural hearing loss. The potential benefit of surgery in these patients is questionable, according to Wielinga and Kerr. Myringosclerosis, by itself, seldom causes any hearing impairment; however, when the TS is located in the epitympanum, it is common that the malleus head and the body of the incus are fixed. In such a case it may be surgically difficult to free them from the tympanosclerotic plaques. It is not uncommon to see fixation of the stapes footplate to the walls of the oval window, and sometimes even the tendon of the stapes can be calcified. When the tympanosclerotic plaques are located on the promontory, the stapes may have to be mobilized to make a space for the surgical procedure that, of course, is risky because the inner ear can be traumatized. Sometimes, the plaques form where earlier bone destruction of the promontory has occurred. In view of this, there is a risk of penetration into the labyrinth when surgery is performed. When the plaques are located near the facial nerve, special precaution is needed. There is no definite curative treatment except surgery. Recurrences are frequent, often resulting in poor hearing.

A successful model for producing acute otitis media in the rat has been developed in Sweden. Our hypothesis is that there might be a factor(s) triggering an immunological reaction sequence that eventually leads to formation of calcified plaques. It would be useful to identify these factors to predict who will be at risk for developing TS, as well as to prevent or even treat the disease. In previous animal studies, the inflammatory response in the rat middle ear has been analyzed using polyclonal and monoclonal protein-specific and cell-specific antibodies. To our knowledge, this inflammatory response has not been analyzed in human subjects. This study was performed to investigate the presence of macrophages, T cells, and B cells in the human middle ear, including the TM. These cell types are commonly seen in inflammatory response. Antibodies against these cells were used in the study as well as antibodies against inducible nitric oxide synthase (iNOS) and interleukin 6 (IL-6). Inducible nitric oxide synthase is an enzyme, expressed in activated macrophages and known...
to produce nitric oxide (NO) that causes vasodilation and kills pathogens. Interleukin 6 is a cytokine that acts as a mediator between different immunocompetent cells. It is also thought to participate in the maturation of cells into osteoclasts, which is a cell involved in bone repair. This study compares the presentation of the aforementioned cells and factors in 2 different patient groups: one group with secretory otitis media (SOM), a condition that is known to have a high propensity (expressed as a percentage) to develop into TS, and one group with already established TS.

The number of positive specimens are presented as a percentage of the whole group of biopsy specimens. Macrophages were frequently seen in this study (MAC 387). When staining for MAC 387, all specimens (100%) in the SOM group were positive (Figure 2). The different mediators had various staining patterns that seemed to be equal in the tissues of the 2 patient groups. Staining showed the presence of IL-6 (Figure 3) and iNOS mostly in mucosal surface cells. Macrophages (Figure 4) and B cells (CD20) were identified in the deeper cell layers, that is, deep in the mucosa, in connective tissue, or around sclerotic lesions. The 2 different patient groups had different staining patterns, that is, there was a different frequency of positive specimens from the various stains. A higher number of positively stained specimens was seen in the SOM biopsy specimens for IL-6 (8 of 9 specimens) (Figure 5), macrophages (9 of 9 specimens) (Figure 6), and B cells (7 of 9 specimens) compared with TS biopsy specimens, in which IL-6 was seen in 3 of 11 specimens, macrophages were seen in 5 of 11 specimens, and B cells were seen in 1 of 11 specimens. The TS biopsy specimens more often stained positively for iNOS (7 of 11 specimens) than did the SOM biopsy specimens (1 of 9 specimens). There was only 1 biopsy specimen in the SOM group that stained positively for iNOS. This specimen was obtained from an air-filled middle ear. One biopsy specimen in the SOM group exhibited myringosclerosis. The presence of T cells (CD3) was not seen in any of the specimens despite several staining procedures. The tonsil tissues and lymph nodes used as positive controls all showed distinct staining of the immunocompetent cells, while the negative controls did not show any staining. Data from Table 1 and Table 2, except CD3, which was not detected, were analyzed using the Mann-Whitney test. There were statistically significant differences in MAC 387 (P = .003), CD20 (P = .004), and IL-6 (P = .003), while iNOS was not statistically significant (P = .03).

Biopsy specimens from 2 different patient groups have been investigated in this study: 1 group with SOM, a condition that is known to have a high propensity (expressed as a percentage) to develop into TS, and one group with already established TS. To find out the differences in the presentation of immunocompetent cells and some mediators, an immunohistochemical technique was used. The presentation of macrophages, T cells, and B cells was analyzed as well as 2 specific markers, IL-6 and iNOS, also thought to be involved in bone repair.

Bacterial challenge and release of IL-6 and IL-8 in humans was performed by Larsson et al. In this study, epithelial cells from a human lung carcinoma cell line and human alveolar macrophages from healthy subjects were stimulated with different agents, among others a strain of gram-positive bacteria. The dose-dependent release of IL-6 and IL-8 was studied and found to be elevated. It showed that these cytokines are produced by epithelial cells after stimulation by this group of bacteria that are of the same type that often invade and colonize the middle ear in acute and chronic otitis media.

Messenger RNA of inflammatory cytokines was studied by Hebda et al in rat middle ear mucosal tissue after challenge with Streptococcus pneumoniae. The time-dependent response was examined for the expression of

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Table 1. Results of 9 Biopsy Specimens Obtained From Children With Secretory Otitis Media*

<table>
<thead>
<tr>
<th>Patient No./ Gender/Age, y</th>
<th>Presence of MS</th>
<th>MAC 387</th>
<th>CD3</th>
<th>CD20</th>
<th>IL-6</th>
<th>iNOS</th>
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<tbody>
<tr>
<td>1/F/3</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2/F/4</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3/F/4</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4/F/3</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5/M/9</td>
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<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6/M/8</td>
<td>+</td>
<td>+</td>
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<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7/M/9</td>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>8/M/3</td>
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<td>−</td>
<td>−</td>
<td>−</td>
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<td>+</td>
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<td>+</td>
<td>−</td>
<td>−</td>
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</table>

* There was no purulent effusion in the tympanic membranes in the children who had transtympanic ventilation tubes inserted for the treatment of secretory otitis media. All patients except 1 (patient 7) had serous effusion. MS indicates myringosclerosis; a plus sign, present or positive; and a minus sign, absent or negative.

† IL-6 indicates interleukin 6; iNOS, inducible nitric oxidase synthase.

Table 2. Results of 11 Biopsy Specimens Obtained From Patients Undergoing Tympanoplasty and/or Ossiculoplasty*

<table>
<thead>
<tr>
<th>Patient No./ Gender/Age, y</th>
<th>TS or MS</th>
<th>MAC 387</th>
<th>CD3</th>
<th>CD20</th>
<th>IL-6</th>
<th>iNOS</th>
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<tr>
<td>1/M/55</td>
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<td>−</td>
<td>−</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>5/F/8</td>
<td>MS</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6/F/44</td>
<td>MS</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>7/F/34</td>
<td>MS</td>
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<td>−</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>8/F/29</td>
<td>TS</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>9/F/9</td>
<td>TS</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>10/F/38</td>
<td>MS</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>11/F/54</td>
<td>MS</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* All patients, except 1 (patient 1), had dry tympanic membranes and underwent tympanoplasty. Patient 1 underwent atticotomy. TS indicates tympanosclerosis; MS, myringosclerosis; a plus sign, present or positive; and a minus sign, absent or negative.

† IL-6 indicates interleukin 6; iNOS, inducible nitric oxidase synthase.
selected cytokine genes. They showed that the expression of IL-6 peaked at 24 to 48 hours after bacterial inoculation. In our study, IL-6 expression was shown in a higher percentage of SOM biopsy specimens compared with TS biopsy specimens. This supports the finding that this particular cytokine peaks early and not late in the inflammatory response.

Another mediator that was frequently seen in TS biopsy specimens in this study of patients with chronic otitis media was iNOS. Inducible nitric oxide synthase is 1 of 3 isoforms of this enzyme and is normally absent in macrophages but expressed when activated by cytokines, leading to production of NO, which can cause vasodilation and kill pathogens, for example, bacteria. Nitric oxide is a radical molecule produced by iNOS and is produced in large amounts once iNOS is formed.17 Nitric oxide plays a role in the inflammatory response in the middle ear and also in the formation of TS.

The bonelike material in the tympanosclerotic plaques can be thought of as ectopic bone, produced in the TM

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**Table 3. Antibodies Used in This Study**

<table>
<thead>
<tr>
<th>Antibody*</th>
<th>Specificity</th>
<th>Dilution</th>
<th>Monoclonal or Polyclonal</th>
<th>Producer</th>
</tr>
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<tr>
<td>MAC 387</td>
<td>Human neutrophil granulocytes, macrophages, squamous mucosa epithelium, and reactive epidermis</td>
<td>1:80</td>
<td>Monoclonal</td>
<td>Dako/AS, Copenhagen, Denmark</td>
</tr>
<tr>
<td>CD3</td>
<td>Human T cells, thymocytes, and Purkinje cells of the brain</td>
<td>1:40</td>
<td>Monoclonal</td>
<td>Becton Dickinson Co, Mountain View, Calif</td>
</tr>
<tr>
<td>CD20</td>
<td>Human B cells</td>
<td>1:200</td>
<td>Monoclonal</td>
<td>Novocastra Laboratories, Newcastle upon Tyne, England</td>
</tr>
<tr>
<td>IL-6</td>
<td>Human, rat, and mouse IL-6</td>
<td>1:50</td>
<td>Polyclonal</td>
<td>Santa Cruz Biotechnology Inc, Santa Cruz, Calif</td>
</tr>
<tr>
<td>iNOS</td>
<td>Human iNOS</td>
<td>1:400</td>
<td>Polyclonal</td>
<td>Zymed Labs Inc, San Francisco, Calif</td>
</tr>
</tbody>
</table>

* IL-6 indicates interleukin 6; iNOS, inducible nitric oxide synthase.

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**Figure 1.** A. Tonsil tissue used as a positive control. Cells positive for antibodies against inducible nitric oxide synthase are indicated by arrows. Bar indicates 200 µm. B. Tonsil tissue used as a negative control, where no stainings could be seen. Bar indicates 400 µm.

**Figure 2.** The different biopsy specimens presented as a percentage of positive specimens in each study group. SOM indicates biopsy specimens from patients having secretory otitis media; TS, biopsy specimens from patients having tympanosclerosis; IL-6, interleukin 6; and iNOS, inducible nitric oxide synthase.

**Figure 3.** Staining against interleukin 6 in a biopsy specimen from the group with tympanosclerosis (the same specimen as in Figure 4). The superficial cells (thin arrow) are stained as well as the ciliae of the mucosa (thick arrows). Bar indicates 100 µm.
and in the middle ear. Osteoblasts produce NO, in response to cytokine production, according to a study by Hukkanen et al.\textsuperscript{18} However, cytokine-induced NO release reduced osteoclast activity. Macrophages were frequently seen in our study. Since this kind of cell is thought to be a precursor to osteoclasts,\textsuperscript{14} there is a possibility of differentiation when a signal is given. This could be, for example, a special cytokine or a combination of several mediators.

Several components in the inflammatory response have been analyzed in human middle ear biopsy specimens. The 2 patient groups differed somewhat in antigen presentation. There were more positive specimens for macrophages, B cells, and IL-6 in the SOM biopsy specimens. There were more positive specimens for iNOS in TS biopsy specimens. There was only 1 specimen in the SOM group that stained positively for iNOS. This patient was the only one who had an air-filled middle ear cavity, while all of the others had fluid-filled middle ear cavities. This is interesting because NO is a gas and obviously must have been produced in the middle ear. If the production of NO was enhanced in this specific patient or if it was inhibited in the others is difficult to speculate. Further investigations are needed to distinguish significant differences between the 2 patient groups.

Other differences between the antigen presentation were the location in the tissue. This seemed to be similar in the 2 patient groups. The presence of IL-6 and iNOS was most evident in the superficial layer of the mucosa, such as in the mucosal epithelium. Macrophages in the middle ear tissue from such patients, always being attacked by antigens emanating from the environment. Inducible nitric oxide synthase was frequently shown in these cells, apparently activated macrophages. The superficial mucosal cell layer and the subepithelial tissues were stained in the TS biopsy specimens. In 1 specimen, cilia from the middle ear mucosa stained positive for IL-6 (Figure 1). This staining pattern is in accord with the findings in an other study (M.F., D.B.-S., M.H., Å. Melhus, MD, PhD, and A. F. Ryan, PhD, unpublished data, 1999) in which rat middle ear mucosa was analyzed using antibodies against IL-6. In both species, IL-6 seems to be produced by epithelial cells and immunocompetent cells. In the SOM biopsy specimens obtained from patients with intact TMs and no air in the middle ear, the labeling pattern was reversed, that is, no iNOS was seen.

The TS plaques are seen as bonelike structures macroscopically and during morphologic examination using a light and/or electron microscope. Therefore, one might consider the TS plaque formation an ectopic production of bone. Osteoclasts and osteoblasts produce a variety of cytokines including IL-6.\textsuperscript{19} Therefore, we were interested to analyze this cytokine in our study. It has been shown that IL-6 stimulates bone

\textbf{Figure 4.} Staining with MAC 387 in a biopsy specimen from the group with tympanosclerosis (the same specimen as in Figure 3). The cells are stained deeply in the tissue (arrows). Bar indicates 200 µm.

\textbf{Figure 5.} Staining against interleukin 6 in a biopsy specimen from the group with secretory otitis media (the same specimen as in Figure 6). Positive cells are seen mostly in the surface (arrows). Bar indicates 400 µm.

\textbf{Figure 6.} Staining with MAC 387 in a biopsy specimen from the group with secretory otitis media (the same specimen as in Figure 5). Positive cells are seen deeply in the tissue (arrows). Bar indicates 400 µm.
resorption in vitro and in vivo and that IL-6 induces osteoclastlike cell formation in human bone marrow cultures. Udagawa et al demonstrated that induction of osteoclast differentiation by IL-6 depends on IL-6 receptor expression by osteoblasts, rather than osteoclast progenitors. Pretreatment of co-cultures with dexamethasone was required for IL-6–dependent formation of osteoclastlike multinucleated cells. In a study by Chole et al, osteoclastic resorption in the bullar bone in mongolian gerbils was studied. The air-filled middle ear was pressurized to 10 mm Hg above atmospheric pressure, which leads to increased osteoclastic resorption of the inner surface of the bullar bone and bone formation on the outer surface. This study shows that macrophages seem to be present in both patient groups and, because they may differentiate into osteoclasts, this might happen when they are stimulated by cytokines, among others, IL-6. The osteoblasts are also induced by IL-6. This probably occurs early in the inflammatory process. Interleukin 6 was expressed in the SOM biopsy specimens. Other inflammatory mediators such as NO are probably involved and NO can be produced both by activated macrophages and endothelial cells. Therefore, in a theoretical model, these cells and their inflammatory mediators can interact in the human middle ear, constituting some of the factors involved in a reaction sequence that eventually leads to the formation of TS. It would be clinically useful if a marker identifying patients with a higher risk for TS development could be found, so that these patients could be clinically followed up and perhaps also be treated. To prevent the calcification process, perhaps a stricter indication for ventilation tube treatment could be used. Thus, children with SOM could be treated with ventilation tubes only in one ear when needed. Another possibility is prevention using drugs. Dexamethasone seems to exert an effect on osteoblasts and osteoclasts and could be a useful tool. Fen spiride is another drug that inhibits the development of MS by local administration. Many other options may be found in the future when a more detailed map of early and late TS formation has been obtained.

More positive specimens were seen in the group with SOM regarding the presence of macrophages, B cells, and IL-6 compared with the group with TS. The TS biopsy specimens more often stained positively for iNOS than did the SOM specimens. T cells could not be identified in any of the specimens. The presence of IL-6 and iNOS was revealed by the antibody staining mostly in the surface cells, such as the mucosal epithelium and the subepithelial tissues. Macrophages and B cells were seen in deeper tissues, such as deep in the mucosa, in connective tissue, or around sclerotic lesions.

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