The Collagen Structure of the Tympanic Membrane

Collagen Types I, II, and III in the Healthy Tympanic Membrane, During Healing of a Perforation, and During Infection

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Objective: To analyze the distribution of 3 collagen types in healthy tympanic membranes, during healing of a perforation, and during infection.

Design: Immunohistochemical study of collagen types I, II, and III in the tympanic membranes of healthy rats as well as during healing of a perforation and in the presence of infection with Streptococcus pneumoniae at various time points.

Setting: Laboratory research center at a university hospital.

Results: Type II collagen was a main constituent of the lamina propria of the pars tensa, whereas type I collagen was found mainly in the pars flaccida. Collagen types I and III were found at the insertion to the malleus handle and in the loose connective tissue surrounding the main collagen layer of the pars tensa. After myringotomy, collagen types I and III were found at the perforation border and around dilated blood vessels early in the healing phase. During infection, the collagen layer was thickened and stained strongly for type II collagen. Collagen types I and III were found in the edematous connective tissue around the main collagen layer and around dilated blood vessels. Three months after perforation or infection, all 3 collagens were present in the lamina propria of the tympanic membrane. Extensive amounts of all 3 collagen types were present in the scar tissue in the tympanic membranes of rats that had undergone myringotomy during the presence of acute otitis media.

Conclusions: The lamina propria of the pars tensa is mainly made up of type II collagen, whereas type I collagen is found in the pars flaccida. Thus the fibrous structure of the pars tensa and flaccida is composed of diverse collagen types, which reflects the different physiological properties of these tissues. Collagen types I and III are present in the acute healing phase after myringotomy and infection, and the collagen content of the tympanic membrane is modified during the inflammatory and healing process.

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CUTICLE OTITIS MEDIA (AOM) is a very common childhood disease that usually resolves spontaneously. Despite this, AOM is usually treated with antibiotics with the intention to avoid complications and sequela. Spontaneous rupture of the tympanic membrane (TM) during episodes of AOM is common. Myringotomy, an instrumental perforation of the TM, is sometimes performed when a patient has been diagnosed as having AOM to obtain bacteriological culture specimens for guiding the choice of antibiotic treatment, for the relief of pain and drainage of the bacteria. Myringotomy is also performed to aerate the middle ear cleft in patients with otitis media with effusion, with or without insertion of a transmyringal tube to improve hearing. In otitis-prone children who have had many episodes of AOM, as well as long-lasting episodes of otitis media with effusion, the TM is subjected to inflammatory processes, which result in a continuous break-down of the collagen structure. The mechanical strength of the TM becomes much weaker in areas where the collagen layer is damaged or deficient, and retraction pockets may develop as sequela.

The TM consists of 2 parts: the pars tensa and the pars flaccida. The outer lining of the pars tensa is made up of a keratinizing epithelium, and the middle layer is composed of radiating and circular collagen fibers, which make up a stiff structure referred to herein as the main collagen layer. The inner layer facing the middle ear is a single-layered epithelium. A very thin layer of loose connective tissue is found underneath both the inner and outer epithelia. The pars flaccida is built in a similar way but its connective tissue layer is thicker and has a much looser structure than that of the pars tensa. Vessels, nerve endings, and mast cells are extensively found in the loose connective tissue of the pars flaccida. The collagen fibers are thicker compared with those of the pars tensa and are organized not as a flat layer only but rather in 3 dimensions.
Collagens are the most abundant proteins of the body and are major components of connective tissue. The collagen molecule is made up of 3 polypeptide chains, called α-chains, that are twisted around each other, forming a long, stiff, triple-stranded helical structure stabilized by the small amino acids proline and glycine. These triple-stranded helical structures together form collagen fibrils that are 10 to 300 nm in diameter. These group into larger units, called collagen fibers, that are several micrometers in diameter and thus visible with a light microscope. About 25 different α-chains have been described, each encoded by separate genes and forming about 20 different collagen types, which are characteristic of different tissues. Collagen fibers are of various diameters and are arranged in different patterns in different tissues. Type I collagen is the main collagen of bone, skin, tendon, and newly healed wounds. Type II collagen is thinner and is the typical collagen of cartilage. Type III collagen is found to a great extent in embryonic tissue, healing wounds, and the connective tissue of the skin, blood vessels, uterus, lung, and liver.

In experimental perforation of the TM in animal models, the epithelial layer soon starts to proliferate over the defect in the direction of epithelial migration of the TM, supported by an underlying bed of inflamed connective tissue. The perforations close within 12 days; 3, 4, and 5 weeks; and 3 months after myringotomy. Three animals were humanely killed at 6 specific time points: at 4 and 12 days; 3, 4, and 5 weeks; and 3 months after myringotomy. The third group (9 rats) was anesthetized with methohexital sodium, and through an incision behind the ear, the tympanic bulla was reached and the ears were infected with a 0.05-M suspension of viable Streptococcus pneumoniae type 3 with 10^7 colony-forming units per milliliter. Three animals were humanely killed at 4 and 12 days and at 3 months, respectively, after inoculation. Finally, in a fourth group (3 animals), the middle ears were first inoculated with S pneumoniae type 3, and 3 days after inoculation the TMs underwent myringotomy in the upper rear quadrant of the pars tensa. The animals were humanely killed at 3 months after inoculation to analyze the collagen structure of scar tissue in the TM.

Under intravenous anesthesia with methohexital sodium, the TMs of all remaining rats at each time point were studied with otomicroscopy. The course of infection and progress of healing after myringotomy were recorded. At the time points described in the previous paragraph, rats were humanely killed by an intraperitoneal injection of pentobarbital sodium (Pentobarbitalnatrium; Apoteksbolaget [now Apoteket AB], Umeå, Sweden). The temporal bones were dissected, fixed in 4% buffered formalin solution (pH 7.0), and heated in a microwave oven to 45°C in 30 seconds. The temporal bones were transferred to a phosphate buffer, and the pars tensa and pars flaccida were dissected. The tissue pieces were dehydrated in increasing concentrations of alcohol (once in 70%, 2 times in 93%, and 3 times in 99.5%) and embedded in paraffin. Next, 5-µm-thick sections were cut with a microtome and prepared using immunohistochemical staining techniques. For detection of type II collagen, a monoclonal mouse anti–type II collagen antibody was used (CIID3, prepared by Rikard Holmdahl, MD, PhD, University of Lund, Lund, Sweden). Slides were rehydrated and treated with hyaluronidase (Sigma-Aldrich Sweden AB, Stockholm), rinsed in 0.05M Tris buffered saline (TBS) at pH 7.6, treated with hydrogen peroxide in methanol, and again rinsed in TBS. The slides were then treated with rabbit serum (1:5) in TBS for 15 minutes, and the first antibody, mouse anti–type II collagen antibody (1:1000), was applied for 45 minutes. Normal rabbit serum (1:5) or normal mouse serum (1:350) was used as a control. The slides were rinsed in TBS, and the second antibody, rabbit antimouse (1:400; Dakopatts AB, Alvsjö, Sweden [now Dako, Glostrup, Denmark]), was applied for 30 minutes. An avidin-biotin complex kit (Vectastain Elite; Vector Laboratories Inc, Burlingame, Calif) was applied for 40 min-

**METHODS**

Our aim was to determine which collagen types can be found in the pars tensa and the pars flaccida of the TM of healthy rats as well as during healing of a perforation and in the presence of infection. The collagen layer of the TM in healthy and diseased rat ears was analyzed with immunohistochemical methods with regard to collagen types I, II, and III. The thickness of the collagen fibrils of healthy TMs was measured using electron microscopy.

The animal experiments described in this article were approved by the ethics committee of Umeå University, Umeå, Sweden.

**IMMUNOHISTOCHEMICAL ANALYSIS**

For immunohistochemical analysis, 33 male rats (each weighing about 250 g) were divided into 4 groups (Table). Both TMs were studied in each animal. The first group (3 animals) had healthy TMs. The second group (18 animals) was intravenously anesthetized with methohexital sodium (Brietal; Eli Lilly and Company, Indianapolis, Ind), and the TMs underwent myringotomy in the upper rear quadrant of the pars tensa. Three animals were humanely killed at 6 specific time points: at 4 and 12 days; 3, 4, and 5 weeks; and 3 months after myringotomy. The third group (9 rats) was anesthetized with methohexital sodium, and through an incision behind the ear, the tympanic bulla was reached and the ears were infected with a 0.05-M suspension of viable Streptococcus pneumoniae type 3 with 10^7 colony-forming units per milliliter. Three animals were humanely killed at 4 and 12 days and at 3 months, respectively, after inoculation. Finally, in a fourth group (3 animals), the middle ears were first inoculated with S pneumoniae type 3, and 3 days after inoculation the TMs underwent myringotomy in the upper rear quadrant of the pars tensa. The animals were humanely killed at 3 months after inoculation to analyze the collagen structure of scar tissue in the TM.

**Table. Experimental Design**

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<th>Time Point After Baseline That Rat Was Killed, d</th>
<th>Electron microscopy</th>
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*Data are given as number of rats.
†Thirty-three rats were divided into 4 groups for immunohistochemical studies, and 3 healthy rats were used in electron microscopy studies.
utes, and the staining was developed in diaminobenzidine (Saveen Biotech AB, Malmö, Sweden).

For detection of collagen types I and III, polyclonal rabbit antitrat antibodies were used (Biogenesis Ltd, Poole, England). The slides were rehydrated and treated with hydrogen peroxide, washed with TBS, and treated with trypsin. Normal swine serum (1:20; Dakopatts AB) was applied for 30 minutes. The first antibody, rabbit antirat type I collagen (1:30) or rabbit antirat type III collagen (1:40 and 1:80; Biogenesis Ltd), was applied for 60 minutes. Normal swine serum (1:20; Dakopatts AB) or normal rabbit serum (1:100; Dakopatts AB) was used as a control. For the second antibody, we used swine antirabbit biotinylated antibody (1:400; Dakopatts AB) for 30 minutes. Thereafter, we used the avidin-biotin complex kit and diaminobenzidin as described in the previous paragraph. The staining was made in pairs, with 1 of the immunostainings counterstained with Mayer hematoxylin. Immunostainings as described herein were performed on diverse tissues (lung, liver, skin, cartilage, muscle, tendon, and aorta) to test the 3 antibodies, and the stainings were judged to be specific in the rat.

**ELECTRON MICROSCOPIC ANALYSIS**

Three healthy Sprague-Dawley rats were used for transmission electron microscopy analysis of the thickness of the collagen fibrils of the pars tensa and pars flaccida (Table). Pars tensa and pars flaccida tissue specimens were collected and fixed in 3% glutaraldehyde in cacodylate buffer and rinsed in 1% osmium tetroxide added in cacodylate buffer, dehydrated in acetone, and embedded in an epoxy resin (Polybed; Polysciences Inc, Warrington, Pa). Ultrathin sections (70 nm) were cut with an ultramicrotome and stained with uranyl acetate and lead citrate. The collagen fibrils were photodocumented at a magnification of 50 000 times in the electron microscope and, in addition, magnified 6 times on photographic paper (total magnification, ×300 000). The diameter of the collagen fibrils was measured in millimeters (±0.05 mm) on glossy prints, and the actual thickness of the collagen fibrils was calculated in nanometers.

**RESULTS**

**HEALTHY TMs**

In the healthy TMs (Figure 1), we found type II collagen in the tympanic annulus, at the insertion of the malleus handle, and in the collagen bundle of the lamina propria of the pars tensa. No type II collagen was found in the pars flaccida.

The main collagen layer of the pars tensa did not stain for type I collagen. Type I collagen was found at the malleus handle and in the connective tissue surrounding the dominating type II collagen layer. Staining for type I collagen was weak in the pars tensa and in the tympanic annulus but was abundant in the pars flaccida.

Type III collagen antibody stained the annulus, the insertion at the malleus handle, and the connective tissue surrounding the main collagen layer. No type III collagen was found in the healthy pars flaccida. By electron microscopic analysis, the thickness of the collagen fibrils was measured and calculated to 10 nm in the pars tensa and 30 nm in the pars flaccida.

**MYRINGOTOMY**

On day 4 after myringotomy (Figure 2A), type II collagen was very prominent in the pars tensa. The main collagen layer was clearly distinct from the surrounding edematous connective tissue and the epidermal keratinocyte layer. At the perforation border, which had started to heal, no type II collagen could be found. In contrast, plenty of collagen types I (Figure 2B) and III was found in the healing area along the perforation border and around dilated blood vessels close the malleus handle.

On day 12 (Figure 2C), the perforation had healed. The perforated collagen bundle of the pars tensa showed clear staining for type II collagen, whereas in the newly formed tissue, some specimens showed staining for type II collagen but in most cases there was no staining. At 3 weeks, there was strong staining for type II collagen in the whole connective tissue layer of the thickened pars tensa, and this strong staining remained for up to 3 months (Figure 2D). The old main collagen layer at the former perforation edge did not stain and seemed to be degenerating. At 12 days and for up to 3 months, collagen types I and III were found in the thickened lamina propria of the healed TM.

**INFECTION**

Four days after inoculation with S pneumoniae type 3, we noted edema of the connective tissue layer and proliferation of the epithelial layers of the pars tensa. Type II collagen (Figure 2A) was found in the main collagen layer, as in healthy tissue. Collagen types I and III (Figure 3B) were distinctly present in the edematous connective tissue around the main collagen layer, especially around dilated blood vessels close to the malleus.
handle. During the acute phase of infection, staining for type III collagen was found in the pars flaccida.

On day 12, we noted a great thickening of the collagen layer, which stained for type II collagen, and this condition remained after the infection had resolved (for up to 3 months). Type I collagen was present in the connective tissue around the main collagen layer, but at 3 months, type I collagen was found in the whole lamina propria. At 12 days, there was staining for type III collagen in the main collagen layer of the pars tensa, at the malleus handle, in the fibrous annulus, and in the pars flaccida. At 3 months, there was extensive staining for type III collagen in the whole lamina propria of the pars tensa.

INFECTION AND MYRINGOTOMY

Three months after infection and myringotomy, the TM was thickened and had an amorphous appearance of the connective tissue layer. A distinct collagenous bundle was not present, but all 3 collagen types were extensively found in the scar tissue.
The collagen layer of the TM has previously been studied extensively using electron microscopy. The fibers were found to be small, with a quadrangular cut surface and lacking the typical periodicity of collagen fibers. Therefore, the collagen fibers of the TM were thought to be a special type of collagen. The collagen fiber structure of the annulus and at the manubrium was continuous with and similar to that of the lamina propria of the TM. However, very little is known about the specific types of collagens that constitute the TM. It has been proposed that collagen types I, II, III, IV, and VI would be present in the TM. Assumptions about the collagen types were frequently made because type II collagen is found in the tectorial membrane and collagen types I, III, and VI are found in the skin. The tectorial membrane, however, is part of the inner ear and does not share its embryological origin with the TM. Even though the outer epithelial layer of the TM is continuous with the skin of the external ear canal, the collagens are not necessarily identical. Autoimmunity to type II collagen has been suggested to play a role in Ménière's disease and in otosclerosis. Therefore, the distribution of type II collagen in the ear has been the focus of immunohistochemical studies. In those studies, type II collagen was found in the annulus and in the lamina propria of the healthy TM in monkeys and rodents. Our study confirms this finding.

The lamina propria of the pars tensa is very thin but surprisingly strong. The pars flaccida is thicker and more elastic. The different properties of the 2 tissues with regard to stiffness, elasticity, and strength may indicate that these structures have different functions in middle ear physiology and hearing. Our study shows that the collagen of the pars tensa and the pars flaccida not only differs structurally in thickness and organization but also in the biochemical composition of collagen types. The main collagen layer of the pars tensa is made up of type II collagen. This is a main constituent of cartilage, and, by providing stiffness, type II collagen is a suitable reinforcement for the pars tensa. The pars tensa is very thin yet stiff, with low elasticity, which may be optimal for its function in hearing because of its high sensitivity to sound vibrations and effective transfer of vibrations to the malleus handle.

The collagen structure of the vocal cords, which are specialized for a delicate vibratory function in voice production, has recently been studied. The vocal cords consist of 2 layers of thick type I collagen fibers, between which is a layer of thinner type III collagen fibers arranged in a basketlike configuration that enables the vocal cords to stretch and modulate the frequency of sound under the action of the intrinsic muscles of the larynx. Thus, even though the vocal cords and TM both have key roles in sound production vs transmission, these 2 tissues are not similar in structure.

In the tympanic membrane, collagen types I and III were found in the thin layer of loose connective tissue just underneath the epithelial layers of the pars tensa but were not found within the main collagen layer. Further, more, collagen types I and III were found at the insertion of the pars tensa at the malleus handle and around blood vessels. Type I collagen was also dispersed in the connective tissue of the pars flaccida. It is typically found in bone tissue, around blood vessels, and in the dermis. Type III collagen is found in extensible tissues, and therefore we would expect to find it in the pars flaccida, which is an elastic tissue. However, in the present study, type III collagen was not found in the healthy pars flaccida but was found in the pars flaccida during infection.

During the healing phase, fibroblasts migrate and proliferate in the wound, producing an extracellular matrix rich in collagen types I and III, fibronectin, and proteoglycans. This activity is initiated by cell-to-cell interaction among fibroblasts and inflammatory cells and the release of growth factors and neurotransmitters into the extracellular matrix. The newly formed tissue is dynamically modulated by synthetic and degrading enzymes to achieve optimal function of the tissue; this process is probably enhanced by the TM being subjected to physical force. In the present study, long-term follow-up was at 3 months, and at that time point, collagen types I, II, and III were observed in the scar tissue. The parallel type II collagen fiber arrangement has optimal tensile strength, and fibers arranged in an unordered manner will make up a weak spot in the TM. It is possible, however, that during collagen turnover, the collagen content of the TM will convert to the original type II.

Temporal fascia is usually used as grafting material during myringoplasty. Ultrastructural studies on temporal fascia show an irregular pattern of collagen fibrils. Immunohistochemical staining of temporal fascia has revealed type IV collagen, which is the collagen typically found in basement membranes and would most likely be found beneath the epidermal layer of the TM. However, type IV collagen was not included in the present study. To our knowledge, the presence of type I collagen in the temporal fascia has not been investigated, but as type I collagen is typically found in fascia, we would expect to find it in the temporal fascia as well. Cartilage has also been used as grafting material, as have collagen films using type IV collagen or a mixture of collagens. According to the results presented herein, cartilage or engineered type II collagen grafts would be the logical choice to use during myringoplasty because the TM lamina propria consists of type II collagen. However, after the graft has served as a scaffold over which the epidermal layer can migrate and close the defect and the mesenchymal cells proliferate, the graft is thinned and possibly degraded and replaced when new collagen is produced. The resulting TM strength and risk of perforation might be more dependent on the patient’s healing capacity and middle ear physiology than on the grafting material used. The change of a TM graft over time is an exciting subject for further studies.

In diseased ears, the collagen structure of the TM is disturbed. In patients with chronic otitis media with effusion, the TM may be retracted toward the middle ear. This can result in an atelectatic TM, in which the collagen layer may be absent, resulting in loss of stability and stiffness. Changes in the collagen layer after infections and after myringotomy are seen in patients as well as in animal experi-
ments. After experimental perforation, the TM healed, albeit with thickening of the pars tensa and disorganized collagen fibers.20 This scar tissue was also seen in the present study. When the TM of a rat with a middle ear infection underwent myringotomy, thickening and scar tissue of the TM was emphasized.15 Immunohistological studies of biopsy specimens from patients with adhesive otitis media revealed type III collagen and, to a lesser extent, type I collagen in the granulation tissue.30 This agrees with our results, in which collagen types I and III were found extensively in scar tissue of the TM.

In perforated rat ears described herein, collagen types I and III had already accumulated at the perforation edge by day 4 after myringotomy, and extensive amounts of all 3 collagen types were found in the healed TM at 3 months after myringotomy. The original collagen layer was embedded in the scar tissue, seemingly degenerating. Thus, the newly formed collagen of the healed TM did not develop from the old, original collagen layer. We found that the amount of collagen types I, II, and III was increased during healing of a perforation and after infection, which could indicate that there is an increased production of these collagens. Collagen types I and III were induced at an early stage of healing, and type II appeared later, indicating that types I and III are involved in the initial stage of healing. In scar tissue, all 3 collagen types were extensively found, which indicates that the collagen type and organization were different compared with healthy tissue. Thus, the collagen organization of the TM is modified during the inflammatory stage and the healing process.

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