Analysis of the Hyalinization Reaction in Otosclerosis

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Otosclerosis is a disease of abnormal bone remodeling, which is unique in that it occurs only in the otic capsule of the human temporal bone. Large foci of otosclerosis may penetrate the endosteal layer of the cochlea, resulting in direct contact with the soft-tissue structures of the inner ear. We have previously reported variable amounts of cochlear element degeneration with otosclerotic endosteal penetration, and in that earlier study, the cochlear elements, including the hair cells, ganglion cells, and stria vascularis, were evaluated quantitatively. A reduction in the population of cochlear elements was not related to the extent and location of otosclerotic endosteal involvement, and a reduction in sensorineural thresholds was observed in some, but not all cases. These observations suggest that factors that limit the effect of otosclerotic endosteal involvement on the cochlear elements may be present. Reports on the association between endosteal involvement of the otic capsule with otosclerosis and sensorineural hearing loss have been summarized in a review of the literature.

As early as 1899, Siebenmann speculated that the diffusion of toxic substances produced by otosclerotic bone into the inner ear fluids resulted in hearing loss. Evidence now supports this concept. Hydrolytic enzymes produced by otosclerotic bone have been identified in perilymph and appear to be a factor leading to the degeneration of cochlear sensory elements and sensorineural hearing loss.

Histologic changes occurring in the cochlear soft tissues at the site of otosclerotic endosteal penetration have been descriptively referred to as a hyalinization reaction. The role of the hyalinization reaction in the development of hearing loss is unknown.

**IMPORTANCE** Otosclerotic bone has been observed to penetrate the endosteal layer of the cochlea, resulting in direct contact with the soft-tissue structures of the inner ear. Sensorineural hearing loss has been observed in some, but not all, of these cases. The development of histologic changes occurring in the cochlear soft tissues at the site of otosclerotic endosteal penetration has been descriptively referred to as a hyalinization reaction. The role of the hyalinization reaction in the development of hearing loss is unknown.

**OBJECTIVE** To evaluate the composition of these hyalinized soft tissues using immunostaining techniques.

**DESIGN, SETTING, AND PARTICIPANTS** Retrospective review in a human temporal bone histopathology research laboratory of 3 specimens from patients with endosteal otosclerotic involvement.

**INTERVENTIONS** Evaluation of human temporal bone pathology findings.

**MAIN OUTCOMES AND MEASURES** Human temporal bone sections with endosteal otosclerotic involvement were studied using immunostaining techniques to identify collagen I, chondroitin sulfate, and keratan sulfate deposition in the hyalinization reaction tissue.

**RESULTS** Intense collagen I staining was demonstrated within the hyalinization reaction in an onion-skin-like layered fashion. In addition, dual immunofluorescence-stained sections for proteoglycans revealed both chondroitin sulfate and keratan sulfate deposition in the hyalinized tissue.

**CONCLUSIONS AND RELEVANCE** The tissue of the hyalinization reaction appears to be composed of collagen I, chondroitin sulfate, and keratan sulfate, which are known to act as molecular barriers. This observation suggests that the hyalinization reaction may limit the diffusion of toxic substances produced by otosclerotic bone into the soft tissues and fluids of the cochlea.
and increased connective tissue density. The extent of the hyalinization reaction is greater in cases with more cochlear otosclerotic involvement and is usually more prominent in the spiral ligament. We have previously proposed that this tissue reaction may mitigate the deleterious effect of otosclerotic bone on cochlear function. To investigate the plausibility of this concept, we have evaluated the composition of these hyalinized soft tissues using immunostaining techniques.

**Methods**

Approval for this research was obtained from the institutional review board at the University of Chicago. Three temporal bones with otosclerotic endosteal penetration of the cochlea have been identified in our collection containing 1700 cases. Histopathologic findings and morphometric data from these cases have been reported previously. The images presented in this report are from the right temporal bone of patient 1 (the fifth case in the earlier study), a woman in her 80s with a history of gradual progressive hearing loss resulting in deafness. In this case, extensive otosclerosis involved the anterior otic capsule, posterior otic capsule, and oval window, with endosteal involvement in the basal, middle, and apical turns of the cochlea. Stapes fixation was present, and the cochlear elements were severely degenerated (Table). Patient 2 (the second case from the earlier study) was also a woman in her 80s, with a bone conduction threshold pure tone average (PTA) of 43 dB at 500, 1000, 2000 Hz and otosclerosis involving the anterior and superior oval window of the right temporal bone. She also had endosteal involvement of the basal turn. Stapes fixation was present, and the cochlear elements were moderately degenerated (Table).

Patient 3 (the first from the earlier study) was a woman in her 50s with a bone conduction threshold PTA of 18 dB and otosclerosis involving the anterior otic capsule of the left temporal bone. She also had extensive endosteal involvement of the second and apical cochlear turns. Stapes fixation was not present, and the cochlear element populations were predominately normal (Table).

The techniques used herein for processing human temporal bones have been previously reported. These specimens were embedded in celloidin. Serial sections of the temporal bones were cut in the horizontal plane at a thickness of 20 μm. Every tenth section was mounted on a glass slide and stained with hematoxylin-eosin for light microscopic examination. Additional adjacent tissue sections were studied using immunostaining techniques.

The antigen retrieval and immunostaining techniques used in this report have also been previously described. The results of staining in control human tissues with known antigen distributions and antibody inhibition studies have confirmed the specificity of these protocols. Images of normal hearing control temporal bones without otosclerosis appear in these reports.

Immunofluorescence staining for type I collagen was performed using a primary rabbit polyclonal antibody against human type I collagen at a 1:50 dilution (Abcam Inc). This primary antibody was detected with a secondary donkey antirabbit IgG antibody conjugated to CY2 at a 1:50 dilution (Jackson Immuno Research Laboratories Inc).

A dual immunofluorescence staining technique was used for the simultaneous detection of chondroitin sulfate and keratan sulfate proteoglycans. The primary antibodies included an IgM isotype monoclonal antibody clone CS-56 at a 1:400 dilution (Santa Cruz Biotechnology) used to detect keratan sulfate proteoglycans. The secondary antibodies were a DyLight 649-conjugated AffiniPure Goat Anti-Mouse IgG at a 1:200 dilution (Jackson Immuno Research Laboratories Inc).

The immunostained sections were examined by confocal microscopy using a disk scanning unit spinning disk confocal inverted IX81 microscope (Olympus). Image data were collected within the dynamic range of the camera (16-bit data per channel) to avoid pixel oversaturation. Fluorescence emission from collagen I-stained structures appear green due to the CY2 fluorophore, which emits visible green light. Tissue autofluorescence appears yellow. Fluorescence emission detected from the chondroitin sulfate DyLight 649 fluorophore was assigned the color green, and fluorescence emission detected from the keratan sulfate DyLight 594 fluorophore was assigned the color red for visual interpretation of the micrographs.

In standard 20-μm-thick sections, structures at different levels will require small focus adjustments for optimal viewing. Therefore, all of the images were sharpened using the Image J program Gaussian Blur and Unsharp Mask tools. Image flare was improved using the Deconvolution Laboratory Richardson-Lucy plugin (http://bigwww.epfl.ch/algorithms/deconvolution/).

<table>
<thead>
<tr>
<th>Patient No./Age, y</th>
<th>LOEI</th>
<th>SF</th>
<th>PTA</th>
<th>CED</th>
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<td>Deaf</td>
<td>Severe</td>
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<tr>
<td>3/50 A, M</td>
<td>Absent</td>
<td>18</td>
<td>Minimal</td>
<td></td>
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</table>

Abbreviations: A, apical turn; B, basal turn; CED, cochlear element degeneration; LOEI, location of otosclerotic endosteal involvement; M, middle turn; PTA, pure tone average bone conduction audiometric threshold in decibels; SF, stapes fixation.
Results

The hematoxylin-eosin staining and immunofluorescence staining results were similar in all 3 cases studied. Images of the tissue from patient 1 have been selected for photographic presentation in this report because the robust hyalinization reaction in this case allowed a clear interpretation of the staining characteristics, which is more difficult where the hyalinization reaction is thinner. Hematoxylin-eosin-stained sections demonstrated a hyalinization reaction in the soft tissues of the cochlea adjacent to areas of direct exposure to otosclerotic bone (Figure 1). The hyalinized tissue appeared pink and was characterized by decreased cellularity and increased connective tissue density. The reaction was more prominent in the spiral ligament than in the soft tissues exposed to otosclerotic bone in the scala tympani and scala vestibuli. The width of the hyalinization reaction was variable, but it was generally thicker in areas with greater otosclerotic endosteal involvement. At the margins of otosclerotic endosteal penetration, where otosclerotic bone approximates normal endosteal bone, the hyalinization reaction was thinner. Breaches in the hyalinization reaction were not observed.

Confocal microscopy of immunofluorescence-stained sections for collagen I demonstrated intense brightness (green, arrowheads) within the hyalinization reaction in an onion-skin-like layered fashion (Figure 2). Yellow, representing autofluorescence in collagen I–staining tissue, predominated in bone and was limited in soft tissues including the hyalinization reaction. The pattern of specific collagen I staining is distinct from the autofluorescence detected. Confocal microscopy of dual immunofluorescence-stained sections for proteoglycans revealed both chondroitin sulfate and keratan sulfate deposition in the hyalinized tissue (Figure 3). Autofluorescence in unstained control temporal bone tissues was negligible at the detection parameters used in the dual proteoglycan immunostaining microscopy protocol.
The histologic analysis of temporal bone tissues demonstrates an incidence of asymptomatic or nonclinical otosclerosis ranging from 8% to 12%. When a focus of otosclerosis is present in the oval window niche, stapes fixation commonly occurs resulting in a conductive hearing loss. This condition is referred to as clinical otosclerosis. The term cochlear otosclerosis has been used to describe similar but different conditions. The first condition is simply when otosclerotic bone has replaced part of the endosteal bone of the cochlea without regard to hearing function. The second condition involves penetration of the endosteal layer that has caused sensorineural hearing loss. The third condition is sensorineural hearing loss resulting from endosteal penetration in the absence of stapes fixation. The first 2 patients described in the present report meet the criteria for the second definition of cochlear otosclerosis. The third patient described in this report meets the criteria for the first definition of cochlear otosclerosis. There were no cases meeting the criteria for the third definition in our collection.

Our own investigations have demonstrated variable amounts of cochlear element degeneration and varying sensorineural thresholds, which were not related to the extent or location of endosteal involvement. Surprisingly, 1 patient with extensive endosteal involvement exhibited normal hearing and predominantly normal cochlear elements. These observations suggest the presence of factors limiting the effect of otosclerotic endosteal involvement on the integrity and function of the cochlear elements.

The nature of the tissue involved in the hyalinization reaction observed adjacent to otosclerotic bone penetration of the cochlear endosteum has long been a subject of speculation. The hyalinized tissue has a scarlike appearance consistent with a reparative process after injury to the soft tissues. Collagen I is known to be a major component of scar tissue. Subsequent evaluation of the hyalinization reaction in our laboratory with polarizing light microscopy demonstrated strong birefringence suggestive of collagen I deposition, which is known for this property due to the highly coagulated structure of its molecules. Collagen I is present in most tissues of the body, where it serves to provide structural integrity and limit tissue permeability. Proteoglycans serve a similar functional role and therefore were also selected for study. Dense collagen and proteoglycans are known to provide a barrier to the migration of molecules and fluids. The chemistry of these barrier properties has been studied experimentally. These studies support the idea that the tissue of the hyalinization reaction may provide limited protection of the delicate inner ear structures from potentially toxic substances produced by otosclerotic bone tissues with a high turnover rate.

A review of publications concerning the association of otosclerotic endosteal involvement and sensorineural hearing loss reveals contradictory concepts regarding the pathophysiologic characteristics of the disease process. Few articles have discussed the significance of the hyalinization reaction. However, 2 similar studies have reported the elevation of bone conductive audiometric thresholds in a group of temporal bones with hyalinization of the spiral ligament compared with a group without hyalinization. Also, stria vascularis atrophy was observed to be most severe on segments of the spiral ligament with the greatest degree of hyalinization. In another report describing observations of temporal bones with otosclerotic endosteal penetration, the width of spiral ligament hyalinization was measured at 1 point in each cochlear turn of a midmodiolar section. The width of the spiral ligament was observed to be thinner in areas with hyalinization but not significantly different from uninvolved areas. A correlation between spiral ligament hyalinization and stria vascularis atrophy was observed in 1 turn of the cochlea, the posterior middle turn, but not in other turns.Bone-conduction thresholds at 2000 and 4000 Hz were significantly associated with the amount of stria vascularis atrophy, but hearing was not associated with spiral ligament hyalinization. Interestingly, decreased immunostaining for enzymes involved with active ion transport in the spiral ligament and stria vascularis was observed in sections with cochlear otosclerotic endosteal involvement.

The findings in these reports suggest that the factors that elicit the formation of the hyalinization reaction also contribute to the development of stria vascularis atrophy and hearing loss, but the findings do not indicate that the hyalinization reaction is the cause of cochlear injury and dysfunction. Although these reports and observations in our own laboratory demonstrate that in most cases the hyalinization reaction does not prevent ultimate harm to the cochlear structures and the development of hearing loss, they do not rule out the possibility that the hyalinization reaction provides limited protection of the cochlear tissues and delays damage.

The observation that the hyalinization is generally thicker in areas with greater otosclerotic endosteal involvement and thinner at the margins of involvement suggests that the hyalinization reaction continues to develop over time as areas of endosteal penetration expand. Given that breaches in the hyalinization reaction were not observed, the margin of endosteal penetration may be the site at which potential toxins enter the cochlear soft tissues. Cases with a more rapid or efficient hyalinization reaction would then be less vulnerable to develop sensorineural hearing loss. The limited number of cases in the present report does not allow conclusions in this regard. However, future studies that correlate a more robust hyalinization reaction in small areas of otosclerotic endosteal involvement with better sensorineural hearing thresholds may provide further support for a role of the hyalinization reaction in mitigating the effect of otosclerotic endosteal penetration. The characteristics of interest would include its density, thickness, and continuity in covering the endosteal areas penetrated. These observations might explain the variable nature of the sensorineural hearing loss associated with cochlear endosteal penetration.

If additional studies support a role for the hyalinization reaction in limiting sensorineural hearing loss, then an improved understanding of the triggers that elicit the development of the hyalinization reaction and the mechanism by which the hyalinization reaction progresses is needed. This information may provide an approach to determine an individual's abil-
ity to respond to otosclerotic endosteal penetration and subsequently lead to the development of strategies that promote a more efficient hyalinization response to stabilize hearing in these individuals.

Conclusions

The nature of the soft-tissue hyalinization reaction observed adjacent to areas of otosclerotic bone penetration of the cochlear endosteum has been explored herein using immunostaining techniques. Intense collagen I staining was demonstrated within the hyalinization reaction in an onionskin-like layered fashion. In addition, dual immunofluorescence staining for proteoglycans revealed both keratan sulfate and chondroitin sulfate deposition in the hyalinized tissue. The presence of these molecules in the hyalinization reaction, which are known to act as molecular barriers, may limit the diffusion of toxic substances produced by otosclerotic bone into the soft tissues and fluids of the cochlea.

REFERENCES


