Distribution of Type IV Collagen in the Cochlea in Alport Syndrome

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Objective: To determine the distribution of α1, α3, and α5 chains of type IV collagen in the cochlea in Alport syndrome.

Design: Case-control study.

Patients: Two patients with sensorineural hearing loss due to Alport syndrome. Both patients had known mutations in the COL4A5 gene.

Main Outcome Measures: Immunostaining was used to study the distribution of type IV collagen (α1, α3, and α5 chains) within the cochlea. Immunostaining was also performed in the cochlear tissues of an unaffected individual used as a control.

Results: In the control ear, α1 staining was observed in the basement membrane overlying the basilar membrane, in the basement membrane of cochlear blood vessels and Schwann cells, and within the spiral limbus. In the control ear, we also observed strong staining for α3 and α5 chains in the basement membrane overlying the basilar membrane and within the spiral ligament. In both cases with Alport syndrome, no immunostaining was observed for α3 or α5 chains within the cochlea, whereas α1 staining was present in locations similar to that seen in the control ear.

Conclusions: The results indicate that isotype switching does not occur within the cochlea in Alport syndrome. The results are also consistent with the hypothesis that the sensorineural hearing loss in Alport syndrome may be due to alterations in cochlear micromechanics and/or dysfunction of the spiral ligament.


Alport syndrome, an inherited progressive renal disease with sensorineural hearing loss (SNHL) and ocular lesions, is caused by mutations in the genes (COL4) that code for the α3, α4, or α5 chains of type IV collagen.1-3 The disease is genetically heterogeneous with X-linked, autosomal recessive and autosomal dominant forms. Most cases (approximately 85%) of Alport syndrome are X-linked and are due to mutations in the COL4A5 gene located on Xq22 that codes for the α5 chain of type IV collagen. More than 300 mutations of the COL4A5 gene producing X-linked Alport syndrome have been reported.

Type IV collagen is the major structural component of basement membrane (BM) and it consists of 3 α chains, which make a triple-helical molecule.4 The trimer composed of α1 and α2 chains (α1-α2-α1) is present ubiquitously in BMs throughout the body. On the other hand, trimers composed of α3, α4, and α5 (α3-α4-α5) or α5 and α6 (α5-α6) chains are restricted in expression to specific BMs of the kidney, inner ear, eye, and other organs.

In Alport syndrome, the glomerular BM within the kidney shows a characteristic ultrastructural abnormality consisting of diffuse thickening and splitting of the lamina densa.5 During normal development, α1 and α2 chains predominate in the glomerular BM. As glomerular maturation progresses, the α3, α4, and α5 chains become the predominant type IV collagen chains in glomerular BM, a process referred to as isotype switching.2,3,5-7 In most males with Alport syndrome, this isotype switch does not occur, as a result of which the α3, α4, and α5 chains are usually absent, as determined by immunostaining. The resulting glomerular BM in Alport syndrome is believed to be defective in structure and function, causing hematuria early in life and progressing to proteinuria and renal failure.

In contrast to renal studies, investigations of the human inner ear in Alport syndrome have lagged behind because of several factors:

1. It is not feasible to take a biopsy sample from the cochlea during life without causing severe SNHL.
2. There is a paucity of human postmortem temporal bone material because temporal bones are not removed or studied as part of routine autopsies.

3. When temporal bones are removed and studied after death, tissue alterations are inevitably induced by postmortem autolysis.

4. The human temporal bone contains the delicate membranous labyrinth within an extremely dense shell of bone, which imposes serious technical challenges in processing temporal bone tissue in a manner that allows for preservation of morphology and the ability to immunostain for various proteins.

In a recent study,

Knowledge of the distribution of various chains of type IV collagen in the cochlea may help in the understanding of the pathogenesis of SNHL at a cellular level. There is a single extant study that examined the distribution of type IV collagen in the normal human inner ear. In the study by Kleppel et al.,

Expression of α1 chains was observed in the BM overlying the basilar membrane (ie, the BM of cells of the organ of Corti), in the BM of cochlear blood vessels, and in the spiral limbus and in the BM of Schwann cells. In contrast, expression of α3 and α4 chains was restricted to the BM overlying the basilar membrane. The study did not examine expression of α5 chains.

To our knowledge, no study has described the distribution of type IV collagen within the cochlea in Alport syndrome. Both patients had known mutations in the COL4A5 gene due to Alport syndrome. Both patients had known mutations in the COL4A5 gene resulting in Alport syndrome. Results indicate that there is selective distribution of α3 and α5 chains within the cochlea, including within the collagen bundles in the spiral ligament and limbus. Results in the cases with Alport syndrome suggest that isotype switching for collagen does not occur in the cochlea, similar to what has been reported in the kidney. The implications of our findings with respect to the pathogenesis of SNHL in Alport syndrome are discussed herein.

METHODS

CASE 1

Patient 1 was a man aged 57 years with X-linked Alport syndrome caused by COL4A5 mutation. This individual had a single-base mutation converting a cysteine to serine at position 1564 in the COL4A5 gene. His epidermal BMs lacked expression of the α5 chain of type IV collagen. He had bilateral progressive SNHL that began in his 20s, and he wore hearing aids for the remainder of his life. His last audiogram at age 53 years showed a bilateral, symmetric, relatively flat, 60- to 70-dB SNHL with good speech discrimination. He died at age 57 years from end-stage renal disease, and both temporal bones were removed 4 hours after death. The left temporal bone was examined by light and electron microscopy, and findings were reported previously. The right temporal bone was fixed in 10% neutral buffered formalin, decalcified using EDTA, trimmed so that only the inner ear remained, and was then embedded in paraffin. Serial sections were cut at a thickness of 8 µm. A representative (mid modiolar) section was stained with hematoxylin and eosin (H&E), while other sections through the cochlea were immunostained.

CASE 2

Patient 2 was a man aged 31 years with X-linked Alport syndrome caused by COL4A5 mutation. This individual had a deletion of exons 43-47 of the COL4A5 gene. He had bilateral SNHL since the age of 9 years and wore amplification for several years. His last audiogram at age 25 years showed a downsloping 60- to 110-dB SNHL on the right with speech discrimination of 56% and a 60- to 90-dB SNHL on the left with speech discrimination of 90%. He died at the age of 31 years from pancreatitis and complications of renal failure, and both temporal bones were removed 10 hours after death. The right temporal bone was used in the present study. The specimen had been stored in 10% formalin for a number of years. The bone was prepared by decalcification using EDTA and trimming so that only the inner ear remained, followed by embedding in paraffin and sectioning at a thickness of 8 µm. A mid modiolar section was stained with H&E; while immunostaining was performed on other sections.

CASE 3

Case 3 involved a woman aged 57 years with no otologic symptoms (control case). This individual died as a result of a hemorrhagic cerebral infarct with pulmonary thromboembolism. Both temporal bones were removed 5.5 hours after death, fixed in 10% formalin, and processed in a manner identical to that described for cases 1 and 2.

Before proceeding with immunostaining on human temporal bones, we conducted experiments using temporal bones of CBA mice to optimize the protocol for immunostaining. The temporal bones of CBA mice were fixed in 10% formalin, decalcified in EDTA, embedded in paraffin, and sectioned in a manner similar to the processing used for human tissues. Antibodies against the α1, α3, and α5 chains of type IV collagen were used to immunostain the animal material to optimize the protocol. The optimized protocol is described in the following section.

IMMUNOSTAINING

Monoclonal antibodies directed against the α1, α3, and α5 chain of collagen IV were used (Wielisa kit; Wieslab AB, Ideon, Lund, Sweden). We did not have access to antibodies against the α2, α4, and α6 chain of collagen IV, and hence our study was restricted to the α1, α3, and α5 chains. Selected slides were deparaffinized in xylene, rehydrated in decreasing strengths of ethyl alcohol, followed by distilled water, and rinsed in a 0.01M phosphate-buffered saline (PBS; pH 7.3). Each slide was treated with 0.0025% protease XXIV solution (Sigma, St Louis, Mo) and incubated at 37°C in a closed humidity chamber (20 minutes for α3 and α5 and 40 minutes for α1; the protease was kept at −20°C and thawed just before use). The protease was drained and slides were rinsed 3 times in PBS. Slides for α3 were incubated in cold glycine-urea solution for 5 minutes and then
IMMUNOSTAINING

Hematoxylin-eosin staining was used to study the inner ears of 2 patients with SNHL due to Alport syndrome and of an unaffected individual used as a control. The findings in the representative mid modiolar sections studied from each temporal bone and stained with H&E are described in the following sections.

RESULTS

LIGHT MICROSCOPY WITH HEMATOXYLIN-EOSIN

Hematoxylin-eosin staining was used to study the inner ears of 2 patients with SNHL due to Alport syndrome and of an unaffected individual used as a control. The findings in the representative mid modiolar sections studied from each temporal bone and stained with H&E are described in the following sections.

Control Ear

The cochlea consisted of 2½ turns. An organ of Corti was present throughout, although it was difficult to differentiate cells (eg, hair cells and supporting cells) because of poor preservation associated with processing in paraffin. The stria vascularis showed mild patchy atrophy, and the cochlear neuronal cells showed a mild loss in the basal turn (compared with normal newborns). The spiral ligament showed partial atrophy in especially the middle and apical turns. All the above changes were consistent with age-related change.

Case 1

The cochlea consisted of 2½ turns with diffuse endolymphatic hydrops of a moderate degree in all turns. There was a zone of separation between the basilar membrane and overlying organ of Corti, similar to the changes observed in the opposite ear (published previously, case 1). While the organ of Corti was present in all turns, its morphologic features were suboptimum owing to paraffin embedding, and it was not possible to determine whether hair cells were missing and whether there was cellular infilling within the tunnel of Corti and extracellular spaces of Nuel (as was observed in the contralateral ear of this individual). The stria vascularis showed mild patchy atrophy, the cochlear neurons showed mild loss in the basal turn, and the spiral ligament showed partial atrophy in especially the middle and apical turns. The cochlear vasculature was normal. All these changes were consistent with age-related change.

Case 2

The cochlea consisted of 2½ turns. There was no endolymphatic hydrops. There was mild patchy atrophy of the stria vascularis, mild to moderate loss of cochlear neurons in the basal turn, and a separation between the basilar membrane and organ of Corti in many turns. Again, suboptimum morphologic features of the processed tissues precluded assessment of cellular architecture within the organ of Corti. Blood vessels supplying the cochlea appeared normal.
Table. Results of Immunostaining for Type IV Collagen in the Cochlea

<table>
<thead>
<tr>
<th>Tissue</th>
<th>α1 Control</th>
<th>α1 Case 1</th>
<th>α1 Case 2</th>
<th>α3 Control</th>
<th>α3 Case 1</th>
<th>α3 Case 2</th>
<th>α5 Control</th>
<th>α5 Case 1</th>
<th>α5 Case 2</th>
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<tbody>
<tr>
<td>BM over basilar membrane</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>2+</td>
<td>0</td>
<td>0</td>
<td>2+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Collagen bundles in spiral ligament</td>
<td>1+ (Basal turn)</td>
<td>1+ (Basal turn)</td>
<td>1+ (Basal turn)</td>
<td>2+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BM of blood vessels</td>
<td>1+</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BM of Schwann cells</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
<td>2+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stria vascularis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spiral limbus</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
<td>2+</td>
<td>0</td>
<td>0</td>
<td>2+</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Reissner membrane cells</td>
<td>2+</td>
<td>1+</td>
<td>2+</td>
<td>2+</td>
<td>0</td>
<td>1+</td>
<td>2+</td>
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<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: BM, basement membrane; 0, negative; 1+, positive; 2+, strongly positive.

α3 Chain of Type IV Collagen

There were striking differences between the control ear and the 2 specimens with Alport syndrome. In the control ear, strong staining was observed in the BM overlying the basilar membrane and in collagen bundles within both the spiral ligament and spiral limbus, with less intense staining in the BM of Schwann cells. No staining was seen in the BM of the cochlear vasculature or in the stria vascularis. Staining was also observed within the cells comprising the Reissner membrane. In both cases of Alport syndrome, there was a general absence of staining in the cochlea using the α3 chain antibody. In case 2 (but not in case 1), there was positive staining of Reissner membrane cells in some turns (Table and Figure).

α5 Chain of Type IV Collagen

The results for the α5 chain were similar to those seen for the α3 chain. The control ear showed strong staining within the BM overlying the basilar membrane, within the spiral ligament and spiral limbus, and within the Reissner membrane cells. No staining was observed in the control ear for Schwann cells or blood vessels. When sections of the control ear were processed after preabsorption of the antibody using α5 type IV collagen antigen, no staining was seen within the cochlea. No staining was noted for the α5 chain antibody in both cases 1 and 2 (Table and Figure).

To our knowledge, the distribution of isotypes of type IV collagen within the human inner ear in Alport syndrome has not been previously reported. We had the unique opportunity to immunostain cochlear tissues from 2 men with Alport syndrome caused by mutations in the COL4A5 gene. We hypothesized that the cochlear tissues of both individuals would show absence of isotype switching, similar to what occurs in renal tissues.2 If our hypothesis were correct, we would expect to find positive immunostaining for the α1 chain antibody, but no staining for the α3 and α5 chain antibody.

The initial step in our study was to examine the distribution of the α1, α3, and α5 chains in an unaffected control ear. As might be expected, α1 expression was seen in many cochlear tissues, including the BM overlying the basilar membrane, the BM of Schwann cells, the BM of blood vessels, and extracellular matrix of the spiral ligament and spiral limbus. These results for the α1 chain antibody were similar to those published by Kleppel et al.9 Immunostaining for the α3 and α5 chains in the control ear was seen only in selected tissues, namely, the BM over the basilar membrane, collagen bundles within the spiral ligament and spiral limbus, and within Reissner membrane cells. No staining was seen within BMs of blood vessels or within the stria vascularis. While expression of α3 (and α4) type IV collagen within the BM of the basilar membrane and within the spiral limbus was also reported by Kleppel et al.,9 the strong staining with the spiral ligament seen in the present study was not observed in the study by Kleppel et al.9 The positive immunostaining with the spiral ligament and other tissues disappeared when the tissue was preabsorbed with α5 type IV collagen, indicating that the observed staining was real. Furthermore, a search of GenBank’s nonredundant protein database for the non-collagenous NC1 domain of COL4A5 revealed no known homology to any other gene, lending further credence to the notion that the α5 staining within the spiral ligament was not a cross-reaction to a similar gene product. We are unsure how to interpret the positive staining for α3 and α5 within the Reissner membrane. Unlike other cochlear tissues, the cytoplasm of the cells appeared to be stained, rather than the BM or extracellular matrix.

Both cases with Alport syndrome were the result of a COL4A5 mutation. The epidermal BMs of case 1 lacked expression of the α5 chain of type IV collagen. In case 2, deletion of exons 43 through 47 of the COL4A5 gene would be expected to prevent formation of α3-α4-α5 trimers that would be stably expressed in the BM. The cochlear sections of both cases showed similar results. There was virtual absence of staining for the α3 and α5 chains, whereas positive α1 staining was seen in various tissues similar to the control ear. These results are consistent with the hypothesis that isotype switching did not occur within the cochlea in both of these individuals with Alport syndrome.

The role of various isoforms of type IV collagen within the inner ear (or other tissues) remains unknown at present. Basement membranes have diverse biological functions such as maintaining tissue architecture, embryonic development, and tissue remodelling.2,3,9,11,12 The

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The presence of α3 and α5 type IV collagen within the BM of the organ of Corti and within the spiral limbus must confer special properties to these structures that allow them to function properly. What these special properties might be remains to be elucidated.

The pathophysiologic mechanisms of SNHL in Alport syndrome remain unknown. In the past, temporal bone studies had failed to identify consistent histopathologic abnormalities within the cochlea. Our team’s recent study of the otopathologic changes in a series of 9

Figure. Results of immunostaining within the cochlea for antibodies against α1, α3, and α5 chains of type IV collagen for the control ear, as well as cases 1 and 2 with Alport syndrome. Expression of α1 is similar in the control ear and both cases with Alport syndrome, being present in the basement membrane (BM) overlying the basilar membrane, the BM of blood vessels, and the BM of Schwann cells. Faint staining is also seen in some collagen bundles within the spiral ligament (SpL). Staining in the control ear for α3 and α5 collagen is similar, with strong staining in the BM over the basilar membrane and in collagen bundles with the spiral ligament. The α5 staining in the control ear disappears when the tissue is preabsorbed with α5 type IV collagen antigen, indicating that the observed staining is real. The BM of blood vessels does not stain for α3 or α5 chains. Both cases of Alport syndrome show lack of α3 and α5 staining. We are unsure how to interpret the positive staining in the Reissner membrane (RM). When examined with high-powered magnification, the cytoplasm of RM cells appeared to be stained, rather than the BM or extracellular matrix (unlike other cochlear structures). OoC indicates organ of Corti; SV, stria vascularis.
cases of Alport syndrome showed 2 characteristic histopathological abnormalities: a separation between the basilar membrane and the overlying BM of the organ of Corti and cellular in-filling of the tunnel and extracellular spaces of the organ of Corti.8 We had hypothesized that these abnormalities result in SNHL by altering cochlear micromechanics. The present study shows that the BM of the organ of Corti contains α3 and α5 chains of type IV collagen in the normal control case. In Alport syndrome, both α3 and α5 chains are absent and the BM contains only the α1 chain. We believe that this defective BM permits normal cochlear function early in life but results in architectural and functional abnormalities over time, leading to SNHL. For example, the BM may fail to provide adequate adhesion between cells of the organ of Corti and the basilar membrane so that basilar membrane motion is not properly tuned by the outer hair cells. Such a hypothesis is consistent with clinical observations that speech discrimination usually remains excellent in Alport syndrome and the SNHL generally does not exceed 60 to 70 dB.17,18

The finding that α3 and α5 are present within the normal spiral ligament but absent in Alport syndrome also enables one to propose an alternative hypothesis for the SNHL. It has become increasingly clear in recent years that the spiral ligament plays an important role in regulating the metabolic and ionic milieu of the inner ear.19-21 The fibrocytes of the ligament are richly endowed with a variety of proteins and enzymes and coupled together by gap junctional systems that permit recycling of potassium as well as other (unknown) functions. Thus, it is conceivable that lack of the normal α3, α4, and α5 isotypes of type IV collagen leads to dysfunction of the ligament, with loss of endolymphatic potential or other abnormalities in the ionic content of endolymph, in turn resulting in dysfunction of hair cells within the organ of Corti. A mechanical dysfunction of the spiral ligament as the cause of SNHL in Alport syndrome has been hypothesized by Harvey et al,22 using a canine model of X-linked Alport syndrome caused by a nonsense mutation of the COL4A5 gene. The authors suggested that loss of the α3, α4, and α5 network within the spiral ligament would reduce tension on the basilar membrane leading to hearing loss. We note that at the light microscopic level, neither the 2 cases in the present study nor any of the cases in our group’s previous study8 showed morphologic evidence of abnormalities or degeneration within the spiral ligament that was greater than what was expected for age.

Additional studies will be needed to ascertain the possible mechanisms of hearing loss in Alport syndrome. Both hypotheses (abnormalities of cochlear micromechanics or spiral ligament dysfunction) would predict that evoked otoacoustic emissions would be abnormal or absent in ears with hearing loss due to Alport syndrome because functional outer hair cells are necessary for otoacoustic emissions.16 A potential method of differentiating between the 2 hypotheses would be to measure endolymphatic potential. A normal endolymphatic potential in the presence of SNHL would argue against metabolic dysfunction of the spiral ligament as being responsible for the hearing loss. Although it is not possible to measure endolymphatic potential in humans, it is feasible to make these measurements in animals.

These findings underscore the need for a valid animal model for hearing loss in Alport syndrome. While several canine and murine models of Alport syndrome have been described,23 the reported phenotypes have not demonstrated hearing loss or the characteristic cochlear histopathologic abnormalities seen in humans. No hearing loss was found in a mouse model of autosomal Alport syndrome,12 and these mice showed significant thickening of BMs surrounding blood vessels in the stria vascularis, a finding that was not seen in the human cases.8 In the canine model, Harvey et al22 reported no hearing loss between affected and unaffected animals up to the age of 5 months. One affected dog tested at age 7 months showed a threshold shift of 25 to 40 dB, which was thought to be perhaps due to hypocalcemia or uremia. The authors did not comment on the morphologic features of the organ of Corti in the affected dogs, especially the separation between the BM and basilar membrane and cellular in-filling within the tunnel of Corti that have been observed in humans.

In conclusion, in the normal human cochlea, α1 type IV collagen was present ubiquitously in BMs, while expression of α3 and α5 chain was restricted to the BM overlying the basilar membrane and to the collagen bundles within the spiral ligament and spiral limbus. In the 2 cases with X-linked Alport syndrome due to mutations in the COL4A5 gene, the distribution of α1 type IV collagen was similar to that seen in a control ear, whereas there was absence of staining for α3 and α5 chains. The results indicate that isotype switching does not occur within the cochlea in Alport syndrome. The results are also consistent with the hypotheses that the SNHL in Alport syndrome may be due to alterations in cochlear micromechanics or dysfunction of the spiral ligament.

Submitted for Publication: June 14, 2005; accepted July 7, 2005.

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Author Contributions: Dr Merchant had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Disclosure: None.

Funding/Support: We are grateful to Axel Eliasen for his generous support of this work. This work was also supported by the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Md (Drs Kashlan and Kalluru), the Curtis L. Atkin Memorial Fund (Dr Gregory) and the National Institute of Deafness and Other Communications Disorders National Temporal Bone, Hearing and Balance Pathology Resource Registry, Boston, Mass.

Acknowledgment: We thank Derin Wester, PhD, for providing the audiometric data for case 2.
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