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Cochlin expression in vestibular endorgans obtained from Meniere's disease patients

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Abstract

The distribution of cochlin and its associated basement membrane proteins (collagen IV, collagen II, laminin- β 2, and nidogen-1) was evaluated in the vestibular endorgans of subjects with Meniere's disease and compared to normal specimens. Cochlin mRNA expression in vestibular endorgans from Meniere's disease specimens was also investigated. Specimens were obtained from patients with Meniere's disease who underwent ablative labyrinthectomy. Control specimens were obtained from both autopsy specimens with documented normal audiovestibular function and from patients undergoing labyrinthectomy for acoustic neuroma excision. In the normal control specimens, cochlin immunoreactivity was found evenly distributed in the stroma of the cristae ampullaris and maculae of the utricle. In Meniere's specimens, cochlin immunoreactivity was markedly increased, which was associated with an increase in cochlin mRNA expression on real-time RT-PCR. Collagen IV and laminin- β 2 immunoreactivity was significantly decreased in Meniere's specimens. Nidogen-1 and collagen II immunoreactivity were unchanged in Meniere's specimens when compared with normal specimens. Cochlin upregulation has been implicated in the hereditary audiovestibulopathy, DFNA9. The increased expression of cochlin and decreased expression of collagen IV and laminin in Meniere's disease are suggestive that overexpression of cochlin may contribute to the dysfunctional inner ear homeostasis seen in Meniere's disease.

Keywords

cochlin; basement membrane; Meniere's disease; inner ear; vestibular endorgans

Introduction

Meniere's disease (MD) consists of episodic vertigo, fluctuating sensorineural hearing loss (SNHL), and tinnitus accompanied by a sensation of aural fullness (Baloh 2001). Episodes

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are known to precipitate from factors such as emotional stress, hormonal fluctuations and salt intake; patients have fluctuating SNHL which progresses throughout their life and they experience a significant decrease in physical and psychosocial quality of life (Soderman et al. 2002). The underlying pathophysiology behind MD remains elusive; prominent theories include decreased endolymph resorption, inner ear vascular abnormalities, autoimmune factors, and an imbalance of water equilibrium in the inner ear (Coelho and Lalwani 2008).

Basement membrane pathology has recently been reported in MD. Our prior study demonstrated that surgically acquired vestibular endorgans in patients with intractable MD exhibit diffuse basement membrane (BM) thickening, which significantly correlated to monolayer degeneration of the horizontal cristae ampullaris and caloric paresis (McCall et al. 2009). Mammalian BMs are intertwined networks of extracellular matrix (ECM) molecules consisting of polymeric laminin and type IV collagen bridged by non-covalent interactions with nidogens and proteoglycans (Kalluri 2003). The mammalian inner ear is rich in BMs, and alterations of the BMs are proposed to mediate the pathology of hearing disorders including Alport's syndrome and presbycusis (Cosgrove et al. 1998; Gratton et al. 2005; Meyer zum Gottesberge and Felix 2005; Sakaguchi et al. 1997; Zehnder et al. 2005).

The BM has been studied extensively in the kidney where it plays a critical role in solute and ion transport regulation (Tryggvason and Wartiovaara 2001). It is conceivable that BM pathology may result in a dysfunctional regulation of endolymphatic fluid and water homeostasis in MD. This has been suggested by the finding of altered expression of aquaporin-4 and 6 (water channels) in vestibular endorgans from MD patients (Ishiyama et al. 2010).

The immunohistochemical distribution of BM proteins in normal human temporal bones was recently described; collagen IV α 2, laminin- β 2, and nidogen-1 colocalized within all human cochlear and vestibular BM's (Ishiyama et al. 2009). These proteins were expressed in the perivascular and perineural BM's in zones demarcating endolymph from perilymph, suggestive of BM involvement in the regulation of water and ionic homeostasis (Ishiyama et al. 2009).

Characterization of the BM and ECM protein expression in the human inner ear may provide clues to the underlying pathophysiology of MD. The most abundant ECM protein in the human inner ear is cochlin, which accounts for 70% of inner ear proteins (Ikezono et al. 2001). Cochlin was found to colocalize with type II collagen in the same ECM areas of the cochlear duct, cochlea and vestibule in studies of rat cochleas, suggesting a role for cochlin in cross-linking to type II collagen fibers (Hosokawa et al. 2010; Mizuta et al. 2008). In addition, Nagy et al. demonstrated that the second vWFA-like domain of cochlin has an affinity for types I, II, and IV collagen (Nagy et al. 2008). Type II collagen is believed to be responsible for the fibrous structure of the inner ear ECM in order to maintain structural stability in the cochlea and vestibule (Slepecky et al. 1992). Type IV collagen provides the supra-structural foundation of the mammalian BM (Kalluri. 2003).

Cochlin, which is the product of the COCH gene, has been associated with the hereditary autosomal dominant form of SNHL, DFNA9 (Khetarpal 2000). DFNA9 deafness is

clinically manifested as a progressive audiovestibular dysfunction with adult onset associated with mutations in the cochlin gene, COCH. Inner ear structures in DFNA9 exhibit eosinophilic microfibrillar deposition and severe atrophy of fibrocytes and neural degeneration of the inner ear (Khetarpal 2000; Robertson et al. 2006). Cochlin expression has not been observed in neuroectodermal structures; thus, it is hypothesized that a disruption of inner ear ionic balance secondary to cochlin deposition occurs in the spiral ligament and spiral limbus (Robertson et al. 2006). Additionally, cochlin overexpression has recently been suggested as an etiologic factor in Usher syndrome type 1F, an autosomal dominant syndromic form of audiovestibulopathy (Chance et al. 2010). Based on these findings, a primary or secondary effect on cochlin and BM protein expression and distribution could impair the normal biochemical ionic and fluid homeostasis of the inner ear, resulting in audiovestibular dysfunction.

The present study addresses the hypothesis that cochlin expression is altered in MD patients. We present the quantitative immunohistochemical and mRNA analyses of cochlin expression in vestibular endorgans from MD patients compared to control specimens obtained from patients undergoing excision of acoustic neuromas (translabrynthectomy approach) and autopsy. The distribution of ECM proteins collagen IV, collagen II, laminin- β 2, and nidogen-1 in relation to cochlin was also studied.

Materials and methods

Autopsy Specimens

The Institutional Review Board (IRB) of UCLA has approved this study. IRB approval number 10-001449, approval date 01/08/2012. Appropriate informed consent for inclusion in the study was obtained from each temporal bone donor before death. The temporal bone donors in this study were part of a National Institute of Health (NIH) funded Human Temporal Bone Consortium for Research Resource Enhancement through the National Institute on Deafness and Other Communication Disorders (NIDCD). The vestibular endorgans were microdissected from one temporal bone from each donor. Human temporal bone specimens were obtained post-mortem from five subjects with a history documenting a lack of auditory or vestibular symptoms (3 female and 2 male, age ranging from 83 to 98 years old, average age = 87). Postmortem time ranged from 8 to 12 hours. The macula utricule and cristae (horizontal canal cristae) from these patients were used for quantitative immunocytochemistry.

Vestibular endorgans obtained from surgery

The utricular maculae and cristae ampullaris were surgically obtained from both patients with intractable Meniere's disease who had elected to undergo transmastoid labyrinthectomy and patients without MD undergoing transmastoid excision of acoustic neuroma. The sensory epithelium was carefully dissected and removed en bloc intraoperatively. All patients had recurrent vertigo spells despite medical management, and had nonfunctional hearing in the operated ear (defined as word discrimination score less than 20%). All patients met the 1995 AAO-HNS criteria for definitive MD (Committee on Hearing and Equilibrium 1995). The 5 patients with MD (3 females and 2 males) had ages ranging from

66 to 84 years old (average age = 76). The macula utricule and cristae (horizontal canal cristae) from these patients were used for quantitative immunocytochemistry.

Tissue processing for immunohistochemistry

Immediately after removal at surgery for MD and acoustic neuroma control specimens, and immediately after microdissection for autopsy specimens, the vestibular endorgans were immersed in 4% paraformaldehyde in sodium phosphate buffer (0.11 M, pH 7.4) and kept in this solution for 16 hrs. Thereafter, the fixative was removed and the utricles were washed with phosphate buffered saline solution (PBS, 0.1M, pH 7.2). Vestibular endorgans from surgery and autopsy were immersed first in sucrose 30% (in PBS) for 3 days, and then embedded in Tissue Tek (O.C.T. compound, Polysciences, Warrington, PA). Twenty-micron-thick cryostat serial sections were obtained using a Microm-HM550 Cryostat. The sections were mounted on Superfrost Plus glass slides (Fisher Scientific, Pittsburgh PA, USA) and stored at -80°C until their use.

Immunofluorescence staining

Cryostat sections were rehydrated with PBS for 10 min and incubated at room temperature for one hour with a blocking solution containing 1% bovine serum albumin fraction-V (Sigma, St. Louis, MO) and 1% Triton X-100 (Sigma, St. Louis, MO) in PBS. Next, the solution was removed and the tissue sections were incubated with cochlin (affinity purified) goat polyclonal antibody raised against a peptide mapping the c-terminus of cochlin from human origin (also recognizes cochlin from mice and rats) (cat # D-19, from Santa Cruz Biotechnology, Santa Cruz California). By western blot of mouse eye tissue extract the antibody gives a single band of approximately 56K. Mouse monoclonal antibodies against collagen IV α 2 (1:1000, Cat # MAB1910, Chemicon, Temecula, CA), collagen II (1:200, Cat # sc-59958, Santa Cruz California), rabbit polyclonal nidogen-1 (1:500, Cat# 481918, Calbiochem), and rat monoclonal laminin- β 2 (1:250, Cat # 05-206, Upstate) were also used. Tissue sections were incubated overnight at 4°C in a humid chamber. The secondary antibodies against goat labeled with Alexa 594 and mouse labeled with Alexa 494 (1:1000 in PBS, Invitrogen Carlsbad, CA) were applied and incubated for one hour at room temperature in the dark. At the end of the incubation, sections were washed again with PBS and covered with a water-soluble mounting media (Vectashield, Vector Labs, Burlingame, CA).

Indirect immunocytochemistry (HRP-DAB)

Frozen cryostat sections were thawed, washed with PBS, and endogenous peroxidase quenching was performed by incubation for 10 minutes with 3% hydrogen peroxide (diluted in 100% in methanol). Slides were then washed with PBS for 10 minutes. Sections were incubated for one hour with a blocking solution containing 5% normal goat serum (Vector Labs) and 0.5% Triton X-100 (Sigma) in PBS. Incubation with the cochlin antibody was performed for 48 hours at 4°C in a humid chamber. The sections were washed with PBS (3×5 minutes). Next, sections were incubated for one hour with biotinylated secondary antibody, goat anti-rabbit polyclonal IgG (1:1000, Vector Labs). Afterward, sections were washed with PBS (3×5 minutes). Next, incubation was performed for one hour with Vectastain Elite ABC reagent (Vector Labs) followed by two $\times 5$ minutes PBS washes.

Immunoperoxidase staining was performed using Immimpact DAB solution (Vector Labs). The reaction was stopped with PBS washes (3×5 minutes) after 2 minutes. Slides were mounted with Vectamount AQ aqueous mounting media (Vector Labs) and glass cover slips.

Immunohistochemical controls

As positive controls, cryostat sections from mouse inner ear were incubated with cochlin antibodies, and human kidney frozen sections were incubated with antibodies against collagen IV, collagen II, nidogen, and laminin- β 2. Inner ear and kidney sections were processed in an identical fashion as human vestibular endorgan sections with regard to fixation time, embedding, sectioning and immunostaining. As negative controls, vestibular endorgans cryostat sections obtained from surgery or autopsy were stained in the same way without the primary antibody or with antibodies that had been pre-absorbed with the corresponding blocking peptide used to generate the cochlin antibody. Antibody absorption was conducted by incubating the primary antibody with the blocking peptide 1 μ g/1 μ g. The mixture was then placed at 37°C for 1 hour after which immunohistochemical staining was done as described above. In both cases, only a faint background in human utricular sections was observed. Both the positive and negative immunohistochemical controls was done as previously described (Ishiyama et al. 2009).

Image acquisition

Immunostained tissue sections were viewed and imaged with an Olympus BX51 fluorescent microscope (Olympus America Inc, NY, USA) equipped with an Olympus DP70 digital camera. The following dichroic mirrors and filters were used: For DAPI, a dichroic mirror (DM) DM40, excitation filter BP330-385 and barrier filter (B) BA420, for Alexa 488, DM505, excitation filter BP470-495, BA510IF, and for Alexa 594, DM570, excitation filter BP530-550, and BA575IF. For bright field a U-MBF3 mirror was used.

To provide unbiased comparisons of the immunoreactive signal between each specimen, all images were captured using strictly the same camera settings. Images were acquired using MicroSuite™ Five software (Olympus America Inc). All images were prepared using the Adobe Photoshop™ software program run in a Dell Precision 380 computer.

Quantification of immunostained areas

Quantitative immunocytochemistry of the cristae and macula utricle cryosections obtained from Meniere's disease, or normative post-mortem patients was performed as described by Ishiyama et al. (2010). To minimize bias in the analysis, the observer was "blinded" to the identity of the tissue samples to be analyzed. A second person not blinded to the sample identity, coded each sample. The immunostained area for each cochlin, collagen IV, collagen II, nidogen and laminin was measured in these three different regions of the sensory epithelia. The area immunostained in the crista and utricular macula sections was quantified by using the computer image analysis software ImageJ (available free over the Internet: <http://rsb.info.nih.gov/ij/index.html>) with the protocol described by Ishiyama et al (2010). Specifically, we measured the total immunostained area underneath the sensory

epithelia which includes stroma, fibrocytes, perineural, perivascular membranes and the BM underneath the sensory epithelia.

Statistical analysis

For each specimen, mean values of the immunostained area were averaged and subjected to one-way repeated measures ANOVA analysis. Comparisons were between MD and normative post-mortem specimens. Five utricles and five cristae per type of specimen were used for quantification. Values were considered statistically significant at $p = 0.05$. Results were expressed as the percentage from the utricles and cristae from normal subjects obtained at autopsy postmortem. The Sigma Stat 3.1 software program (Sigma Stat, Ashburn, Va., USA) was used for statistical analysis.

Considerations on the use of vestibular endorgans obtained from autopsy and surgery for immunohistochemistry

Autolytic artifact secondary to fixation delay is unavoidable in human material. This effect is especially critical for examination by cytochemical techniques (Ishiyama et al. 2009). To minimize post-mortem artifacts, temporal bones were harvested between 3 and 9 hours post-mortem. In general, shorter post-mortem times are associated with better signal and a lower background. In the surgical specimens, upon surgical extraction, tissue is immediately fixed and transported to the laboratory and specimens are post-fixed for 24 hours.

Real time RT-PCR

For cochlin mRNA expression, the utricular maculae specimens were obtained surgically from 3 patients with MD (2 males, ages 66 and 77 years old and 1 female, age 60 years old) and 3 patients undergoing translabyrinthectomy for acoustic neuroma excision (2 males, ages 60, 73 years old and 1 female, age 59 years old) as controls. Specimens were received in RNAlater (Ambion, Texas) and taken immediately to the laboratory where they were placed overnight in 4° C. The following day, specimens were placed at -80° C until mRNA was extracted.

Total RNA and reverse transcription was extracted from each utricle using TRIzol Reagent (Invitrogen) as described by Ishiyama et al. (2010) The samples were stored at -20°C until use. Amplification was performed with the single-color real time PCR system in a Stratagene Mx3000P as described by Ishiyama et al (2010) Cochlin primers: Reference sequence accession # NM_004086; Catalog number PPH08287A, Coagulation factor C homolog, cochlin (*Limulus polyphemus*) were validated to ensure amplification of a single product with appropriate efficiency. Samples were run in duplicate and the amplified products were analyzed by 4% agarose gel electrophoresis.

The threshold cycle (C_t) for each reaction (which is directly related to the amount of starting template in the reaction) was measured within the exponential phase of the PCR reaction using Stratagene software MxPro V3. Data were normalized using the housekeeping reference gene GADPH. A difference in C_t values (DC_t) was then calculated for each gene by taking triplicate C_t values from three reactions and subtracting the mean C_t of the triplicates for the reference gene (GADPH) for each cDNA sample at the same

concentration. An additional difference in DC_t value (DDC_t) was calculated for the cochlin gene by taking the triplicate DC_t values for each gene in Meniere's specimens and subtracting the mean DC_t value of the triplicates for acoustic neuroma specimens. The relative expression level was calculated using the 2^{-DDC_t} method (Livak and Schmittgen 2001).

Results

Cochlin immunoreactivity (IR) in human vestibular endorgans

Cochlin-IR was present in the stroma and BM underneath the sensory epithelia of both the crista (Fig 1a) and the macula utricle (Fig 1a') in the normal specimens. The supporting cells and hair cells in the sensory epithelia showed no immunoreactivity. There was a uniform cochlin-IR signal in all stroma regions of the cristae from the planum semilunatum to the central portion. Figures 1b and 1b' shows cochlin-IR (low and high magnification respectively) in the stroma and BM of the maculae utricle from a MD patient. There was strong immunoreactivity when compared with the vestibular endorgans obtained from normal specimens. These findings were consistent in every MD specimen analyzed.

Cochlin mRNA expression in human vestibular endorgans

Isolated mRNA was subjected to real-time RT-PCR to detect the mRNA of cochlin. A comparison of cochlin mRNA expression levels between MD and control (acoustic neuroma) specimens showed a 36% increase in cochlin mRNA expression from the MD cristae (Fig. 2a). The RT-PCR amplified cochlin mRNA products from MD and control specimens (AN) showed bands on electrophoresis between 150 and 200bp consistent with established cochlin mRNA length (Fig. 2b).

Colocalization of cochlin and collagen-IV

Cochlin immunofluorescence (IF) was also examined in both control specimens from postmortem normal subjects and MD vestibular endorgans obtained from ablative surgery. In the normal crista ampullaris, cochlin-IF appeared as a fine punctuated reaction in the stroma and blood vessels (Fig. 3a). In MD crista ampullaris (Figure 3b) there was an increased in cochlin-IF in the stroma and BM underneath the sensory epithelia. Collagen-IV-IF in the normal crista was present in perivascular BM and the BM underneath the sensory epithelia (Fig 3c). In the cristae from MD specimens, collagen-IV-IF was decreased in the BM (Figure 3d), but not in perivascular and perineural BM. Figure 3e and 3f are merged images from the normal (Fig 3a and c) and MD crista (Fig 3b and 3d) respectively.

In the normal macula utricle, cochlin-IF appeared as a fine punctuated reaction in the stroma and blood vessels (Fig. 4a). In MD utricle there was an increased in cochlin-IF in the stroma and BM underneath the sensory epithelia (Figure 4b). Collagen-IV-IF in the normal utricle was present in perivascular BM and the BM underneath the sensory epithelia (Fig 4c). In contrast, in the utricle from MD specimens, collagen-IV-IF was decreased in both perivascular and perineural BM (Figure 3d). Figure 4e and 4f are merged images from the normal (Fig 4a and c) and MD utricle (Fig 4b and 4d) respectively.

Cochlin and Laminin- β 2-IF

Figure 5a shows cochlin-IF in the normal utricle (Fig. 5a). In MD utricle there was an increased in cochlin-IF in the stroma and BM underneath the sensory epithelia (Figure 5b). Laminin- β 2-IF was present in perivascular BM and the BM underneath the sensory epithelia in the normal utricle (Fig 5c). In contrast, in the utricle from MD specimens, laminin- β 2-IF was decreased the BM underneath the sensory epithelia and to a lesser extent in perivascular and perineural BM (Figure 5d). Figure 5e and 5f are merged images from the normal (Fig 5a and 5c) and MD utricle (Fig 5b and 5d) respectively. The cristae ampullaris from MD showed a similar decrease in laminin- β 2-IF (not shown).

Collagen II and nidogen-1 immunofluorescence

In the normal crista and utricle collagen II and nidogen immunofluorescence is localized in perivascular and perivascular BMs and BM underneath the sensory epithelium resembling the distribution of collagen-IV. In MD vestibular endorgans collagen II and nidogen did not changed when compared with the normal vestibular endorgans (not shown).

Quantitative immunocytochemistry

Quantitative immunofluorescence measurements for cochlin showed statistically significant increased cochlin immunoreactivity from the cristae and utricle from subjects with MD compared to those from normal autopsy subjects (Fig. 6). Changes in collagen IV were statistically significantly decreased in MD cristae and utricle when compared to the normal vestibular endorgans (Fig. 6). Laminin- β 2 immunofluorescence was statistically significantly decreased in MD cristae and utricle when compared to the normal vestibular endorgans (Fig. 6). In contrast, no statistically significant changes were observed in collagen II and nidogen immunofluorescence in MD specimens when compared with normal specimens.

Cochlin-IR controls

Positive controls for the cochlin antibodies were tested in mice cochlea (Fig. 7a) and vestibular endorgans (Fig 7b). Immunoreactive signal was detected in the spiral ligament and stroma of the macula utricle of the mouse. Negative controls (human vestibular endorgans incubated with normal serum or no primary antibody) did not show immunoreactivity within the vestibular (Fig 7c) or mouse cochlear tissues (Fig. 7d). Positive and negative controls for collagen-IV, collagen-II, nidogen-1, and laminin- β 2 were conducted as previously described (Ishiyama et al 2010).

Discussion

In the present study we describe the protein immunolocalization and mRNA expression of cochlin, and ECM proteins in the vestibular endorgans from patients diagnosed with MD and compare the cochlin expression to normal human vestibular endorgans. Both immunolocalization and mRNA expression showed increased levels of cochlin deposition and mRNA expression in the BMs of the cristae ampullaris and maculae utricle in subjects with MD. In addition, there was a significant decrease in collagen-IV and laminin- β 2 in MD specimens, while collagen-II and nidogen-1 remained unchanged.

Increased cochlin expression in human MD specimens

Cochlin, the major ECM protein in the human inner ear, is associated with the autosomal dominant form of adult-onset SNHL and vestibular symptoms, known as DFNA9 (Ikezono et al. 2001; Robertson et al. 1998). DFNA9 has a similarity in clinical symptomatology to MD (Fransen et al 1999). Patients with DFNA9 typically manifest with progressive SNHL starting between the third and sixth decade with accompanying vestibular symptoms. Despite the likelihood that the specific COCH mutations causing DFNA9 audiovestibulopathy are not responsible for sporadic MD (Sanchez et al. 2003; Usami et al. 2003), our data demonstrates that there is cochlin overexpression in the vestibular endorgans of the subjects with intractable MD.

The function of cochlin in the inner ear has largely been studied in reference to DFNA9, as the largest component of the inner ear ECM. Mature cochlin is a modular polypeptide consisting of a LCCL domain and two vWFA-like domains, which are found primarily in proteins in the ECM (Colombatti and Bonaldo 1991). Immunohistochemical analysis of human and rodent fetal ears and adult rodent ears showed cochlin expression in the same areas as type II collagen (Goodyear and Richardson 2002; Khetarpal et al. 1994; Tsuprun and Santi, 1999). In the rat vestibular organ, cochlin is expressed in the stroma of the maculae of the otolithic organs and the cristae ampullares, as well as in channels in the bony labyrinth that transmit dendritic innervation to the cristae and maculae, similar to our postmortem normal specimens (Ikezono et al. 2005). COCH mRNA expression has also been detected in the stromal cells underlying sensory epithelium in the crista ampullaris histologically by in situ hybridization (Robertson et al. 2001).

The role of cochlin and the etiology of DFNA9 has been recently studied using a cochlin a knock-out ($Coch^{-/-}$) and a knock-in ($Coch^{+/+}$) mouse model (Jones et al 2011). Both mouse models showed elevated auditory brain responses (ABRs) at 21 moths of age. $Coch^{-/-}$ model showed changes at the highest frequency, and the $Coch^{+/+}$ model showed changes at all frequencies. Heterozygous knock-in mouse model showed elevated ABRs similar to homozygotes. Vestibular evoked potentials were elevated in the $Coch^{-/-}$ model at 13 and 21 months and as early as 7 months in the $Coch^{+/+}$ model. These results indicate that in both mouse models, vestibular function is compromised before the cochlear function (Jones et al, 2011). Morphological examination of the $Coch^{-/-}$ and the $Coch^{+/+}$ mice did not reveal any significant histological changes. These results suggest that pathology may be due to events occurring at the molecular level.

Collagen-IV and Laminin- β 2 expression are decreased, collagen I and nidogen remain unchanged in MD specimens

Cochlin was upregulated in the vestibular endorgans of MD patients, while collagen IV and laminin expression was significantly decreased. Our presented data showing a marked increase in cochlin expression and an associated decrease in collagen IV and laminin expression lends support to BM pathophysiology in MD. Studies of the inner ear ECM support a structural role for cochlin.

Our study does not show alterations in collagen II expression in the vestibular endorgans from MD subjects. The eosinophilic deposits containing cochlin in DFNA9 were associated with a decreased expression of type II collagen, suggesting a direct effect of the mutated cochlin or altered binding of cochlin with the fibrillar collagens (Robertson et al. 2001). In studies of glaucoma, increased cochlin expression followed disease progression and also paralleled a decrease in type II collagen, suggesting altered tissue architecture leading to an obstruction in aqueous flow in the eye (Bhattacharya et al. 2005a, 2005b). Another study used electron microscopy to examine DFNA9 temporal bone sections, and showed normal collagen fibrillogenesis disrupted by excess microfibrillar substance, resulting in the degradation of collagens and ECM components (Khetarpal 2000). Like collagen II, nidogen expression was not altered in vestibular endorgans from subjects with MD.

Altered ECM protein expression in MD

We propose that an altered cochlin and ECM protein expression may play a role in MD. Given that endolymphatic hydrops is the most consistent temporal bone finding associated with MD, it is likely that a disruption in water and ion homeostasis in the inner ear via ECM protein alteration may contribute to the underlying physiology of the disease. ECM molecules are thought to regulate extracellular ion homeostasis and fluid filtration (Eikmans et al. 2003). Our prior work analyzing the histopathology and ultrastructure of vestibular endorgans in patients with intractable MD showed BM thickening, which correlated with neuroepithelial degeneration (McCall et al. 2009).

This study is unique in its analysis of the cochlin expression in individual vestibular endorgans from human patients with active MD. At the time of surgery, these patients exhibited active disease, i.e. recent vertigo spells that were unresponsive to medical therapy. Therefore, they were in an active disease state at the time of surgery. The severity of disease did appear to be generated from the surgically removed endorgans as the vast majority of patients undergoing ablative labyrinthectomy clinically improved postoperatively. Given the complex interaction of cochlin with other ECM proteins in the human vestibular endorgans further studies are necessary to elucidate the role of cochlin in the pathophysiology of MD. It is important to note that there are several types of cochlin isoforms which have been detected in the inner ear (Sekine et al. 2010); changes in protein expression may be heterogeneous, and the current study identifies only one type of cochlin. Further studies investigating other cochlin isoforms are indicated.

In conclusion there was an increase in cochlin expression in the BMs of vestibular endorgans from subjects with MD. There is a marked decrease in collagen IV and laminin- β 2 expression in MD patients. Cochlin appears to be structurally important in BM physiology and its upregulation may contribute to the dysfunctional inner ear homeostasis underlying the pathophysiology of MD.

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Fig. 1. Cochlin immunoreactivity (IR) in normal and MD vestibular endorgans. (a) Cochlin-IR (arrows) was present in the normal crista (autopsy) stroma (ST) and the basement membrane (BM) ({} underneath the sensory epithelia (SE). Supporting cells and hair cells in the SE showed no immunoreactivity. (a') High magnification view of the normal macula utricle, like in the crista cochlin-IR is present in the ST tissue and BM ({}). (b). In MD specimens strong cochlin-IR (arrows) was present in the ST and the BM underneath the macula utricle SE. (b') High magnification view from (b), strong cochlin-IR is seen in the BM and ST. Hematoxylin counterstaining. Magnification bar in a and b is 100 μ m. In a1 and b1 is 25 μ m.

Fig. 2.

Cochlin mRNA expression in MD. (a) COCH expression in MD versus control (acoustic neuroma, AN) specimens. Data represent the mean $\pm$ standard deviation of the three MD and three control cristae. (b) RT-PCR products of COCH amplified from MD and AN patients. Molecular size markers (M) are indicated (ladder amplicon sizes of 150bp, and 200bp).

Fig. 3.

Cochlin and collagen IV immunofluorescence (IF) in normal and MD crista. (a) Cochlin-IF in the normal crista (red color), appears as a fine punctuated reaction in the stroma (ST) (arrows). (c) Collagen IV-IF (green arrows) in the same section (a). Collagen-IV is present in perivascular BM and the BM ({}) underneath the sensory epithelia (SE). (B) Cochlin-IF from a MD crista with increased cochlin-IF (red) in the BM underneath the SE ({}) and stroma. (d) collagen-IV-IF is decreased in the BM but not in the stroma. The SE was not immunoreactive. Fig e and f are merged images from a and c, and b and d respectively. DAPI in blue indicate cell nuclei. Magnification bar in all figures is 60 μm.

Fig. 4.

Cochlin and collagen IV immunofluorescence (IF) in normal and MD utricle. (a) Cochlin-IF in the normal utricle stroma (red color) (arrows). (c) Collagen IV-IF (green arrows) in the same section like (a). Collagen-IV is present in perivascular BM and the BM ({} underneath the utricle SE. (B) Cochlin-IF from a MD utricle with increased cochlin-IF in the BM ({} and stroma. (d) collagen-IV-IF is decreased in both the BM underneath the SE and the stroma. Fig e and f are merged images from a and c, and b and d respectively. DAPI in blue indicate cell nuclei. Magnification bar in all figures is 60 μm.

Fig. 5. Cochlin and Laminin- β 2-IF. (a) Cochlin-IF in the normal utricle stroma (red color) (arrows). (c) Laminin- β 2-IF (green arrows) in the same section like (a). Laminin- β 2-IF is present in perivascular BM and the BM ({}) underneath the utricle SE. (b) Cochlin-IF from a MD utricle in the BM ({}) and stroma. (d) Laminin- β 2-IF is decreased in both the BM underneath the SE and the stroma. Fig e and f are merged images from a and c, and b and d respectively. DAPI in blue indicate cell nuclei. Magnification bar in all figures is 55 μ m.

Fig 6.

Quantitative comparison of cochlin, collagen-IV, collagen-II, nidogen and laminin immunoreactivity between autopsy (green) and MD (red) specimens. Cochlin IR reveals statistically significant changes between MD (increase IR) and normal crista and utricle. Collagen-IV and laminin-IR were statistically significantly decreased. Collagen-II-IR and nidogen-IR showed no significant differences.

Fig. 7.

Cochlin-immunoreactivity (IR) controls. (a) Positive control: Cochlin-IR in mouse cochlea spiral ligament, and (b) the macula utricle. (c) Negative control: Cross-section of the human utricle immunostained with all reagents except for the cochlin antibody; no specific reaction was observed (d) negative control no immunoreactivity in the mouse cochlea was detected when the primary antibody was omitted. SL: spiral ligament, STV: stria vascularis, SE: sensory epithelia, St: stroma. Magnification bar in a, b and d is 35 µm, is C is 25 µm.