Matrix Metalloproteinase Dysregulation in the Stria Vascularis of Mice with Alport Syndrome

Implications for Capillary Basement Membrane Pathology

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Alport syndrome results from mutations in genes encoding collagen $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, or $\alpha 5(\text{IV})$ and is characterized by progressive glomerular disease associated with a high-frequency sensorineural hearing loss. Earlier studies of a gene knockout mouse model for Alport syndrome noted thickening of stria capillary basement membranes in the cochlea, suggesting that the stria vascularis is the primary site of cochlear pathogenesis. Here we combine a novel cochlear microdissection technique with molecular analyses to illustrate significant quantitative alterations in stria expression of mRNAs encoding matrix metalloproteinases-2, -9, -12, and -14. Gelatin zymography of extracts from the stria vascularis confirmed these findings. Treatment of Alport mice with a small molecule inhibitor of these matrix metalloproteinases exacerbated stria capillary basement membrane thickening, demonstrating that alterations in basement membrane metabolism result in matrix accumulation in the stria capillary basement membranes. This is the first demonstration of true quantitative analysis of specific mRNAs for matrix metalloproteinases in a cochlear microcompartment. Further, these data suggest that the altered basement membrane composition in Alport stria influences the expression of genes involved in basement membrane metabolism. (*Am J Pathol* 2005, 166:1465–1474)

Alport syndrome is a relatively common (1 in 5000) heritable basement membrane (BM) disorder characterized by progressive glomerulonephritis associated with high-

frequency hearing loss and retinal flecks.¹ In the renal glomerulus, mutations in either the collagen $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, or $\alpha 5(\text{IV})$ chains result in the absence of all three chains in the glomerular basement membrane (GBM), resulting in an embryonic GBM collagen composition [$\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains only]. Persistence of this abnormal BM composition underlies progressive changes in GBM composition, ultrastructure, and function, culminating in fibrosis and renal failure. Matrix deposits and GBM splitting suggest abnormal homeostasis. Documented alterations in transforming growth factor- $\beta 1$, matrix protein expression by glomerular podocytes,² and the appearance of abnormal laminin isoforms in the GBM of Alport mice, dogs, and humans support this notion.^{3,4}

The Alport mouse model, created by targeted mutagenesis of the collagen $\alpha 3(\text{IV})$ gene, has many of the hallmarks of the human disease.^{5,6} As for X-linked Alport Syndrome, the collagen $\alpha 3(\text{IV})$ knockout mouse lacks expression of collagen $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ chains in GBMs. This useful model has been exploited by many laboratories to explore the mechanisms underlying Alport glomerular and tubulointerstitial pathogenesis,^{3,7–11} which has enhanced our understanding of the disease and lead to the discovery of potential therapeutic options.^{12,13} Inner ear pathogenesis in the Alport model has received less attention, but has been associated with thickening of the stria capillary basement membranes (SCBMs).¹⁴

The stria vascularis is a tissue microcompartment of the cochlea that functions to maintain the high potassium content of the scala media. A high potassium concentration is essential for initiating signaling in cochlear hair cells. The accumulation of matrix associated with SCBM thickening suggests disequilibrium in the mechanisms

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underlying homeostatic maintenance of the SCBM in Alport mice. This is most likely due to altered synthesis or turnover of the SCBM matrix proteins. Matrix turnover is mediated primarily via regulated expression of a family of matrix metalloproteinases (MMPs), a specialized class of enzymes involved in embryonic development, tissue remodeling, inflammation, and disease. We surmised that expression of the MMPs in the stria vascularis of Alport mice might be altered, implicating a role for BM metabolism in SCBM thickening. Most often in diseases in which elevated matrix accumulation is observed, it is accompanied by elevated MMP expression.^{10,15,16} The up-regulated MMPs presumably reflect cellular compensatory mechanisms aimed at limiting the rate of matrix accumulation. To test this hypothesis, we examined the expression and enzymatic activity of MMPs in microdissected specimen of the stria vascularis from both normal and Alport mice. We chose to focus on MMP-2, MMP-3, MMP-7, MMP-9, MMP-12, and MMP-14, because the literature for the 26 member MMP family suggests these are most frequently associated with inflammatory diseases, fibrosis, and tumor invasion.^{10,15,17–20} Hence this subgroup of MMPs is known to be prone to dysregulation associated with human progressive disease processes involving matrix remodeling. Using real-time polymerase chain reaction (PCR), we found significantly elevated transcripts for MMP-2, MMP-9, MMP-12, and MMP-14 (membrane type 1 MMP that activates MMP-2) in Alport stria vascularis relative to controls. Gelatin zymography of extracts from microdissected stria vascularis specimens indicated similarly elevated enzyme activities. Treatment of Alport mice with a small molecule inhibitor for the MMPs exacerbates SCBM thickening, directly implicating an imbalance in the regulation of synthesis and turnover of GBM components as responsible for thickened SCBM in Alport mice. Collectively, these studies show the feasibility of true quantitative molecular analysis of inner ear microcompartments by combining sophisticated dissection techniques with real-time PCR. The data indicate that SCBM pathology is associated with altered matrix metabolism in Alport mice.

Materials and Methods

Mice

Collagen IV $\alpha 3$ knockout mice on the 129 Sv/J background and their normal littermates were used at 7 to 9 weeks of age, before end-stage renal disease. Wild-type littermates served as controls. Mice were bred in-house. The Institutional Animal Use and Care committees of the University of Pennsylvania and Boys Town National Research Hospital approved the animal protocols. Extreme care was taken to minimize pain and discomfort.

Immunogold Localization

For ultrastructural localization of BM proteins, we used a postembedding procedure using Unicryl embedding media (Vector Laboratories, Burlingame, CA). Tissue was

fixed by transcardiac perfusion of animals with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. The cochlea was decalcified and separated from the semicircular canals. The cochlea was then bisected longitudinally (base to apex) through the modiolus with a fine scalpel. The hemicochleae were embedded in Unicryl resin (Vector Laboratories), then cut and stained as described previously.²¹

Blocks were cut at 70 nm and sections collected onto 200-mesh formvar/carbon-coated grids (Electron Microscopic Sciences, Ft. Washington, PA). The grids were floated on the surface of staining solutions. The primary antibodies were optimized by testing a series of concentrations. Optimum dilutions were generally two to three times more concentrated than that used for immunofluorescence analysis (1:5 for anti-type IV collagen; 1:75 for both anti-laminin-1 and anti-laminin $\alpha 5$ antibodies). The primary antibody was added in a solution of blocking buffer containing 1% bovine serum albumin (purified by cold ethanol precipitation), 0.1% Tween-20, and 0.1% fish gelatin in PBS (pH 7.3). Incubation of the primary antibody was performed for 4 hours at room temperature. After six washes in PBS (10 minutes each) at room temperature, an anti-rabbit antibody directly conjugated to 10-nm gold particles (Vector Laboratories), was added (in blocking buffer), and allowed to react for 2 hours at room temperature. Grids were then washed six times (10 minutes each) in PBS at room temperature. The sample was counterstained with uranyl acetate and lead citrate before examination by electron microscopy (Phillips CM-10, Eindhoven, The Netherlands).

Immunofluorescence Analysis

Tissue Preparation

Sedated (Avertin, 300 μ g/g body weight, i.p.) mice were transcardially perfused with 10 ml of PBS followed by 10 ml of fixative. The temporal bones were removed and the cochlea perfused via the round window. The temporal were then immersed in the fixative for 30 to 60 minutes. For all antibodies used in the study with the exception of collagen IV antibodies, the fixative was 4% paraformaldehyde. For anti-collagen IV immunostaining, Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid) was used. After fixation, the cochleae were decalcified (0.12 mol/L ethylenediamine tetraacetic acid, pH 7.0, 2 days, 5°C) and prepared for light or colloidal gold immunohistochemistry.

Light Microscopic Immunohistochemistry

Decalcified temporal bones were frozen in OCT (Tissue-Tek) and cryosectioned (5 μ m) in the mid-modiolar plane. Frozen blocks contained a cochlea from an Alport mutant mouse and a normal control. The tissue sections were serially postfixed in cold acetone and air-dried before analysis by immunofluorescence. For type IV collagen immunostaining sections were denatured (acid urea: 1 hour, 4°C, pH 3.5; or 0.1% sodium dodecyl sulfate: 45

minutes, 37°C) to expose masked epitopes. Antibodies against the NC1 domains of type IV collagen are known not to react well without first denaturing the tissue.⁵ Sections were incubated overnight at 4°C with the primary antibody. The rinsed sections were then incubated (6 hours, 4°C) in the fluorescein isothiocyanate-conjugated secondary antibody. Images were captured using an Olympus BH-2 immunofluorescence microscope configured with a Spot RT digital camera and Image Pro-Plus image analysis software. Final figures were assembled using Adobe Photoshop software.

Antibodies

The antibodies used and their dilutions were rabbit anti-human MMP-2 (Chemicon, Temecula, CA) 1:100 and rabbit anti-human MMP-9 (Chemicon) 1:100 as described previously.¹⁰ Anti-laminin $\alpha 5$ chain-specific antibody, 1:200, a gift from Jeff Miner (Nephrology Division, Washington University, St. Louis, MO), was described previously.^{22,23} The type IV collagen antibody (Southern Biotechnology, Birmingham, AL) 1:20, representing only the $\alpha 1(IV)$ and $\alpha 2(IV)$ chains, was raised against murine type IV collagen purified from Englebreth-Holms swarm tumor cell cultures. Similarly, antibodies against laminin-1 (Sigma Immunochemicals, St. Louis, MO) 1:200, representing the ($\alpha 1\beta 1\gamma 1$) heterotrimer were rabbit antisera raised against Englebreth-Holms swarm tumor-derived laminin (which is only laminin-1). It should be noted that laminin-1 antibodies react with all three chains, and thus recognize the $\gamma 1$ chain. Therefore, this is a pan antibody for laminins containing the $\gamma 1$ chain. The use of the collagen IV and laminin-1 antibodies was described previously.⁵

Isolation of Stria Vascularis

The stria vascularis was microdissected from the remainder of the membranous labyrinth in a cooled Petri dish containing cold Hanks' buffered salt solution (HBSS), pH 7.4. A stereomicroscope with $\times 25$, $\times 40$, and $\times 65$ magnification was used to view the specimen. The temporal bone was isolated from the skull using scissors to transect the skull midsagittally, while blunt no. 3 forceps separated the temporal bone from the anterior fossa and dural venous sinus. The temporal bone was immersed in cold HBSS and excess loose connective tissue removed with no. 5 forceps. The semicircular canal portion of the temporal bone, oriented to view the medial aspect, was held with blunt no. 3 forceps. The tips of another blunt forceps were used to apply force to the apex of the temporal bone, thus separating the cochlea from the more lateral middle ear structures. The tympanic membrane and middle ear ossicles were then removed. Figure 3A shows a cochlea prepared for microdissection of the stria vascularis. The isolated temporal bone was transferred to a dish with fresh HBSS and sterile instruments were used for the strial microdissection. Sterile, blunt forceps were used to hold the semicircular canal portion of the temporal bone. A hole was made into the

apex of the cochlea using sharp-tipped no. 5 forceps. The otic capsule was removed to expose the lateral wall tissues (spiral ligament and stria vascularis) in a spiral manner beginning at the apex and continuing toward the cochlear base for $\sim 3/4$ turn. Forceps were used to bite and chip the bone from the lateral wall using the pigmented strial tissue as a guide. The apical turn of the cochlea was exposed, but was still intact at this point. No. 55 forceps were used to grasp the modiolus of the upper cochlea and pull it out through the apex. Portions of the organ of Corti usually adhere to the modiolus. The stria vascularis of the apical turn was gently teased from the underlying spiral ligament using the no. 55 forceps until it floated freely (Figure 3B). In like manner, the bone was then removed from the lower portion of the cochlea using the no. 5 forceps, followed by the modiolus and organ of Corti. The entire length of stria vascularis was removed from the cochlea with the no. 55 forceps (Figure 3C). A Derlacki capsule knife (V. Mueller, Inc., Toronto, Ontario, Canada) was used to collect and transfer the stria to a microcentrifuge tube containing holding solution for further analysis.

Real-Time PCR Analysis

cDNA was prepared using cells-to-cDNA II kit (Ambion, Austin, TX). Briefly, the stria was lysed, incubated at 75°C, and treated with RNase-free DNase to remove any contaminating genomic DNA before reverse transcription (RT). The lysate from two striae (one mouse) was reverse-transcribed using oligo dT and M-MLV reverse transcriptase. To ensure that the quantitation of MMP transcripts in serial samples was not affected by differences in the amount of RNA added, integrity of RNA, or sample-to-sample differences in levels of RT-PCR inhibition, an internal control reaction targeting the GAPDH gene was run in multiplex with each reaction, and used to normalize results for MMP transcripts. The primers and TaqMan probes for targeted molecules (MMPs) and murine GAPDH were designed and purchased from Applied Biosystems (ABI, Foster City, CA). Fluorescence and quencher abbreviations are FAM, 6-carboxyfluoresceine; MGBNFQ, minor groove binder nonfluorescence quencher.

Sequences of PCR Primers and TaqMan Probes

MMP-2: sense, 5'-GTT TAT TTG GCG GAC AGT GAC A-3', anti-sense, 5'-AGA ATG TGG CCA CCA GCA A-3', and probe 5'-6FAMNFQ-CCA CGT GAC AAG CC-MGB-3'; MMP-9: sense, 5'-CCA AGG GTA CAG CCT GTT CCT-3', anti-sense, 5'-GCA CGC TGG AAT GAT CTA AGC-3', and probe, 5'-6FAMNFQ-ACT CGT GCG CTG CC-MGB-3'; MMP-12: sense, 5'-GCC ACA CTA TCC CAG GAG CAT ATA-3', anti-sense, 5'-AGC TGC ATC AAC CTT CTT CAC A-3', and probe 5'-6FAMNFQ-ATG CAG AGA AGC CC-3' MGB-NFQ; MMP-14: sense 5'-GAG GAG AGA TGT TTG TCT TCA AGG A-3', anti-sense, 5'-GGG TAT CCA TCC ATC ACT TGG TTA-3',

and probe 5'-6FAMNFQ-TCC TCA CCC GCC AGA G-MGB-3'; GAPDH: TaqMan rodent GAPDH control reagents (catalog no. 4308313) containing the primers and VIC-probe were purchased from Applied Biosystems. Accession numbers for primer sequences: MMP-2, NM_008610; MMP-3, NM_010809; MMP-7, NM_010810; MMP-9, NM_013599; MMP-12, NM_008605; and MMP-14, NM_008608.

PCR Conditions

PCR was performed with TaqMan Universal PCR master mix (Applied Biosystems), which contained AmpliTaq Gold DNA polymerase, AmpErase uracil-*N*-glycosylate, dNTPs with dUTP, passive reference, and optimized buffer components. AmpErase uracil-*N*-glycosylate treatment prevented the possible reamplification of carryover PCR products. Each target molecule was co-amplified with primers and TaqMan probe for GAPDH in the same PCR tube. The total volume of the PCR reaction was 50 μ l. The final concentration of each oligonucleotide in the PCR reaction was as follows: GAPDH primers, 100 nmol/L; primers for target molecules, 900 nmol/L; TaqMan probe for GAPDH, 200 nmol/L; and TaqMan probe for the target molecules, 250 nmol/L. Thermal cycling was initiated with incubation at 50°C for 2 minutes and 95°C for 10 minutes for optimal AmpErase UNG activity and activation of AmpliTaq Gold DNA polymerase, respectively. After this initial step, 40 cycles were performed, heating at 95°C for 15 seconds for melting and 60°C for 60 seconds for annealing and extension. All ddH₂O controls were negative for target and housekeeping genes.

The data were analyzed using the comparative threshold cycle (C_T) method. The mRNA quantity for the control is normalized to 1 and all other quantities from Alport samples are expressed as fold difference relative to the controls. No measurable fluorescence signal was detected in repeated RT-PCR runs in which the reverse transcriptase was omitted from the reaction mixture. Primers were tested by standard endpoint RT-PCR, and the single band obtained was sequence verified. Real-time RT-PCR was performed on a TaqMan ABI 7000 sequence detection system (Applied Biosystems).

Gelatin Zymography

Substrate gel electrophoresis (zymography) was performed to identify whether the stria extracts contained MMP activity and to identify the enzymes involved. The two stria from a mouse were homogenized in a total volume of 50 μ l of Tris/saline buffer (50 mmol/L Tris.Cl, pH 7.5, 0.9% w/v NaCl, 0.2% Triton X-100). Twenty μ l of the homogenate was used directly for gelatin zymography. The gelatin-degrading activity was examined by electrophoresis on an 8% sodium dodecyl sulfate-polyacrylamide gel containing gelatin (1.0 mg/ml) without prior heating or reduction of the sample, as previously described.^{11,12} The addition of prestained molecular weight markers (Bio-Rad Laboratories, Richmond, VA) and conditioned media from human HT1080 cells, which

contain MMP-2 and MMP-9, to the gels facilitated identification of the enzymes present in stria vascularis extracts. After electrophoresis, the gels were washed twice for 30 minutes in 2.5% Triton X-100 and incubated in 50 mmol/L Tris-HCl buffer, pH 7.5, containing 0.15 mol/L NaCl, 10 mmol/L CaCl₂, and 0.02% NaN₃ for 16 hours at 37°C. Gels were stained with Coomassie Brilliant Blue R250 and then destained. Gelatinase activity was visualized by negative staining. Gelatinolytic activity of each band was evident as a clear band against the blue background of stained gelatin. The accuracy and sensitivity of the zymographic technique for determining protease levels was analyzed by running different amounts of purified MMP-2 and MMP-9, which confirmed the linearity of zymography (not shown). The clear bands (MMP-2 and MMP-9) were analyzed by computer-assisted densitometric scanning using Imagequant Software (Molecular Dynamics, Sunnyvale, CA).

Administration of MMP Inhibitors

MMP inhibitors were administered between 4 and 7 weeks of age. Three animals were used for each treatment group (saline-injected control, saline-injected Alport, and MMI 270-injected Alport). MMI270 [chemical name: *N*-hydroxy-2-({4-methoxysulfonyl}{3-pocoly})-amino)-3-methylbutanamide] was originally called CGS27023A. The drug was provided to us by NOVARTIS Pharma AG, Basel, Switzerland. All drugs were freshly prepared before administration. MMI-270 was solubilized in 0.9% saline and administered daily by intraperitoneal injection (50 μ g/g body weight). A solution was prepared at 7.5 mg/ml and 200 μ l was administered twice a day by intraperitoneal injection from 4 weeks to 7 weeks of age. MMI270 was chosen for this study because it is the only available MMP inhibitor with potent anti-MMP-12 inhibitory activity. Additional Alport mice and a group of normal controls received injections (200 μ l) of the carrier, 0.9% saline, according to the same schedule.

Transmission Electron Microscopy

Animals were anesthetized (Avertin, 400 μ g/g body weight, i.p.) and transcardially perfused with PBS (5 ml, 23°C) followed by a solution of 4% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L phosphate buffer (10 ml, pH 7.4, 23°C). The temporal bones were removed and opened to expose the otic capsule. The stapes was removed. A perforation was made in the round window after which fixative was perfused for 1 minute through the oval window. The bulla was then immersed in fixative (12 hours, 4°C), rinsed with phosphate buffer, postfixed (2% OsO₄ in 0.1 mol/L phosphate buffer, 20 minutes) and decalcified (80 ml, 120 mmol/L, 2SS ethylenediamine tetraacetic acid, 23°C, 24 hours). The cochleae were dehydrated through a graded series of ethanols and propylene oxide before infiltration and embedding in plastic resin (EmBed 812; EMS, Fort Washington, PA). After polymerization (12 hours, 58°C) the cochlea was bisected in the mid-modiolar plane. The half-cochleae

Figure 1. Colloidal gold immunolocalization in the SCBM of wild-type (+/+) and Alport (-/-) littermates. Tissue was reacted with antibodies specific for the indicated BM proteins (laminin-1 recognizes all $\gamma 1$ chain-containing laminins). Secondary antibodies were conjugated to 10-nm gold particles. **Asterisks** denote the location of SCBMs. Scale bar, 0.15 μm .

were re-embedded and completely polymerized (18 hours, 58°C). Ultrathin sections (70 nm) cut in the mid-modiolar plane of the cochlea were stained with uranyl acetate and bismuth (or lead citrate) and examined in a JEOL JEM-1010 microscope. Digitized images of capillary profiles in the stria vascularis were acquired at $\times 20,000$ using an Orca charge-coupled device camera (Hamamatsu Photonics, Bridgewater, NJ). AMT Advantage 12-HR software (version 5.4.2.239; Advanced Microscopy Techniques, Danvers, MA) was used to make measurements of BM width in at least four locations in each capillary profile (1/quadrant) that represented the typical width of the BM in that quadrant. Efforts were made to avoid taking measurements in focal regions that appeared thinner or thicker than the remainder of the BM for that capillary.

Data Presentation and Statistical Analysis

Data were expressed as the mean and SD. Differences between the means were assessed using Student's *t*-test with Bonferroni adjustment for all data except the BM width measures. The differences in the width of the BM among the treatment groups and their controls were examined using a one-way analysis of variance. Posthoc multiple comparisons were made using the Tukey test. Significance for all of the analyses was set at a probability level of 0.01.

Results

SCBM Thickening in Alport Mice Is Associated with Accumulating Matrix

A colloidal gold immunocytochemical approach was used to examine the relative amounts of specific matrix proteins in the SCBM of normal versus Alport mice. Antibodies specific for either type IV collagen, laminin-1 ($\alpha 1\beta 1\gamma 1$ heterotrimer), or the laminin $\alpha 5$ chain were reacted with ultrathin sections of stria vascularis from either normal or Alport mice. Secondary antibodies were linked to 10-nm gold particles. The results in Figure 1 illustrate

that these matrix proteins are significantly elevated in the SCBM of Alport mice relative to normal mice (as indicated by the higher density of gold particles in the SCBM). Counting the gold particles in multiple stria capillary profiles from triplicate experiments revealed a 4 ± 0.5 -fold increase in type IV collagen, a 2.5 ± 0.4 -fold increase in laminin-1, and a 17 ± 3 -fold increase in laminin $\alpha 5$ in the Alport SCBM relative to controls.

Because the MMPs are a major mediator of BM turnover, we performed immunohistochemical staining on cochlear cryosections using antibodies specific for different metalloproteinases (MMP-2, -3, -7, and -9). No immunostaining for MMP-3 and MMP-7 was observed, while these same antibodies reacted robustly with fibrotic kidney sections from Alport mice (data not shown). Figure 2, A and B, shows MMP-2 immunostaining in the stria vascularis of both normal and Alport mice. MMP-9 expression was not observed in the stria vascularis of normal mice (Figure 2C), but could be detected at the luminal surface of stria marginal cells in Alport mice (Figure 2D). Anti-MMP-12 antibodies did not detect positive immunostaining in either normal or Alport stria due to sensitivity of the antibodies to paraformaldehyde fixation.

Real-Time RT-PCR Analysis of MMP-2, MMP-9, MMP-12, and MMP-14 mRNAs

The results of immunofluorescence immunostaining suggest that MMP-9 protein is elevated in the stria of Alport mice relative to normal mice. Quantitative analysis of specific molecular components in cochlear microcompartments has not previously been attempted due to the inaccessibility and the minute quantity of available tissue. We overcame these obstacles by combining a novel stria microdissection technique with real-time PCR analysis. The microdissection of the stria vascularis from a 7-week-old mouse cochlea is illustrated in Figure 3. This procedure, described in detail in the Materials and Methods section, was used to produce pure preparations of the stria from 10 normal and 10 Alport mice. The microsamples from each animal were reverse-transcribed and

Figure 2. Immunofluorescence localization of MMP-2 and MMP-9 in the stria vascularis of normal and Alport mice. Cryosections from 7-week-old normal (**A** and **C**) and Alport (**B** and **D**) mice were reacted with antibodies specific for either MMP-2 (**A** and **B**) or MMP-9 (**C** and **D**). The stria vascularis (denoted with **arrows**) is strongly immunopositive for MMP-2 in both normal (**A**) and Alport (**B**) sections. MMP-9 immunostaining is only observed in the stria vascularis of Alport mice (**D**) and is confined to the luminal surface of stria marginal cells. Scale bar, 50 μ m.

analyzed blindly for mRNAs encoding MMP-2, MMP-9, and MMP-14. The data obtained were normalized to GAPDH mRNA expression, which was analyzed in multiplex with each sample. The results in Figure 4 illustrate that mRNA levels for MMP-2 are marginally induced (2.7 ± 0.4 -fold) in Alport stria relative to controls. However, mRNA levels for MMP-9, MMP-12, and MMP-14 (MT1-MMP) are markedly induced in the Alport stria (5 ± 0.5 -fold, 9.5 ± 1.2 -fold, and 9.2 ± 1.4 -fold, respectively).

Gelatin Zymography Confirms Elevated MMP Proteolytic Activity in the Stria of Alport Mice

To determine whether elevated mRNA levels translate to elevated proteolytic activity in the Alport stria, we per-

formed gelatin zymography using lysates from microdissected stria. Proteolytic activity of two forms of type IV collagenase (72 kd, MMP-2; 92 kd, MMP-9) in control and Alport stria was analyzed by gelatin zymography. The results in Figure 5, A and C, show that MMP-9 activity is markedly elevated in the stria of Alport mice relative to normal mice, in which the MMP-9 activity was virtually undetectable. MMP-2 activity was only slightly elevated in the stria of Alport mice relative to normal mice (Figure 5B). However, the active isoform of MMP-2 (62 kd) was only observed in Alport stria (Figure 5A, arrowhead). These data are consistent with the results obtained by real-time PCR analysis. Importantly, the data demonstrate that MMP activities are significantly elevated in the stria vascularis of Alport mice.

Figure 3. Strial microdissection. **A:** Pigmented intermediate cells permit a clear view of the stria vascularis (arrows) through the lateral aspect of the bony otic capsule. **B:** The apical turn of the stria vascularis floats freely (arrows) after removal of the otic capsule and detachment from the spiral ligament. **C:** Freed stria vascularis retains the shape of the cochlea coils. rw, round window; ow, oval window; a, apex; b, base.

Treating Alport Mice with MMP Inhibitors Exacerbates SCBM Thickening

If the observed changes in MMP expression reflect the activation of cellular mechanism meant to limit the rate of matrix accumulation in the SCBM, then inhibition of MMP activity should exacerbate SCBM thickening. To test this hypothesis, we treated Alport mice from 4 weeks to 7 weeks of age with the MMI 270, a well-characterized MMP inhibitor with broad substrate specificity that includes MMP-2, MMP-9, MMP-12, and MT1-MMP.^{24,25} Cochleae were removed and analyzed using transmission electron microscopy. Morphometric measurements of SCBMs from at least three different animals revealed significant additional thickening of the SCBM in MMI 270-treated Alport mice relative to thickened SCBM present in the saline-injected Alport mice (Table 1). Thickening was observed throughout the profile view of the SCBM (Figure 6; A to C). No preferred location in the cochlear spiral was noted in that SCBM thickening. In addition, there were regions of focal gross thickening observed in some strial

capillary profiles in which the BM thickness was as much as 500 nm (Figure 6D). These areas clearly demonstrate defects in BM metabolism in MMI 270-treated Alport mice. Measurements reported in Table 1 did not include the focal regions of gross SCBM thickening.

Figure 4. Real-time RT-PCR analysis of MMP mRNA in stria vascularis isolated from control and Alport mice. Stria vascularis was dissected from 10 9-week-old normal and Alport mice. Both stria from each mouse were pooled, the RNA isolated, and subjected to real-time PCR analysis using primers and probes specific for the indicated MMPs. Samples were run blinded and the uncoded results analyzed for statistically significant changes in MMP mRNA expression. A significant up-regulation (*, $P > 0.01$) is noted for each MMP in the stria from the Alport mice. All PCR products were confirmed by DNA sequence analysis.

Figure 5. **A:** Gelatin zymography for MMP-2 and MMP-9 proteolytic activity in stria extracts from normal versus Alport mice. **Lanes 1 to 4,** tissue extracts from normal unaffected mice; **lanes 5 to 8,** tissue extracts from Alport mice. **B and C:** Quantitative analysis of MMP-2 and MMP-9. Cells cultured from a human fibrosarcoma cell line (HT-1080) were used as a positive control (**lane 9**). Bands corresponding to MMP-2 (72 kd) and MMP-9 (92 kd) were scanned and the data analyzed quantitatively. The results shown in **B** and **C** indicate significantly elevated activities for both MMP-2 and MMP-9 in Alport stria relative to normal controls. Note that active MMP-2 (indicated by an arrowhead and plus symbol) is only observed in lysates from Alport stria.

Table 1. Strial Capillary Basement Membrane Width for Alport Mice Treated with MMI-270 or with Saline Compared to Saline-Treated Control Mice

Group	Control saline	Alport saline	Alport MMI270
Mean (nm)	74	101	115
SD (nm)	21	23	39
Max (nm)	138	194	253
Min (nm)	34	47	53
n	59	53	70

Discussion

Cochlear pathogenesis in the Alport mouse model is thought arise from a functional defect in the SCBMs.¹⁴ The defect manifests not only as thickened SCBMs, but also with clear pathology of both the endothelial cells and the marginal cells of the stria vascularis. In this study, we combined a novel strial microdissection technique with quantitative molecular analysis to show that MMP-2 and MMP-9 mRNA and enzymatic activities are up-regulated in the stria vascularis of Alport mice relative to normal mice. Significantly elevated levels of MMP-14 mRNA are also observed in the Alport stria relative to normal mice. MMP-14 is a membrane-bound protein that has been shown to proteolytically activate the proform of MMP-2.²⁶ These observations correlate well with MMP enzyme activities as measured by gelatin zymography.

We also show that thickening of the SCBM is associated with significantly elevated matrix protein deposition using a semiquantitative colloidal gold immunocytochemical approach. These findings are consistent with earlier qualitative studies.¹⁴ Elevated MMP activities may

seem counterintuitive with observations of thickened SCBMs. However the elevated MMP expression is likely the result of a compensatory mechanism activated to limit the rate of matrix accumulation. Such a mechanism has been suggested to explain elevated MMP expression in myocardial fibrosis,²⁷ atherosclerosis,²⁸ and renal fibrosis.^{10,29} Indeed, there is a complex interrelationship between signaling mechanisms that regulate expression of both matrix molecules and MMPs. For instance, many of the cytokines that are known to modulate expression of extracellular matrix during inflammatory responses also influence expression of the MMPs. The most notable of these include transforming growth factor- β , tumor necrosis factor- α , and BMP-7.^{30–33} Expression of these cytokine families has not yet been examined in the Alport stria vascularis.

Integrin signaling mechanisms have also been strongly implicated in modulating expression of both matrix molecules and MMPs.^{34–37} The fundamental difference between normal and Alport stria vascularis is altered composition of BM collagen.¹⁴ Therefore, it is possible that initiation of the SCBM thickening involves altered integrin signaling due to altered availability of specific cell matrix components that serve as ligands for integrin binding and activation. In earlier *in vitro* work, we demonstrated that cultured strial marginal cells express both integrins and BM matrix components.³⁸ This marginal cell line may provide a useful system for exploring the mechanisms underlying matrix-dependent dysregulation of BM homeostasis in disease.

The observation that treating Alport mice with MMP inhibitors exacerbates the SCBM thickening (Figure 6) directly implicates altered BM metabolism in maintaining

Figure 6. Treatment of Alport mice with the MMI 270 exacerbates SCBM thickening. Alport mice were treated from 4 to 7 weeks of age with MMP-270 (C) or saline (B), and the SCBM examined by transmission electron microscopy. **Arrows** denote SCBM. **A**, Untreated control; **B**, saline-treated Alport; **C**, MMI 270-treated Alport; **D**, area of SCBM in MMI 270-treated Alport mouse showing a region with focal irregular thickening. Scale bar, 0.5 μ m.

normal SCBM composition and thickness. It should be noted, however, that our findings do not confirm a mechanistic link between SCBM thickening and hearing loss in the Alport mouse. Wiedauer and Arnold³⁹ found thickened capillary BM in humans with Alport syndrome, however a more recent study of temporal bones from seven Alport patients suggest the SCBM is morphologically normal.⁴⁰ Thickening of the SCBM is also implicated in hearing loss associated with aging,^{41–43} diabetes,⁴³ and autoimmunity,^{44,45} and thus may constitute a major mechanism of progressive hearing loss in humans. Molecular dissection of the mechanisms underlying these observations has been hampered due to the inaccessibility of the tissue, the lack of techniques sensitive enough to provide reliable data, and the lack of cell culture systems that accurately recapitulate *in vivo* observations. Attempts have been made to culture stria cells, suggesting progress in this area is forthcoming.^{38,46} In this study we demonstrate that stria microdissection combined with modern molecular analytical techniques can be used to elucidate the molecular mechanisms underlying progressive SCBM thickening. This combination of techniques provides a useful model for identifying mechanisms underlying pathologies in other relatively inaccessible tissue microcompartments.

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