Increased expression of MMP-2, MMP-9 (type IV collagenases/gelatinases), and MT1-MMP in canine X-linked Alport syndrome (XLAS)

VELIDI H. RAO, GEORGE E. LEES, CLIFFORD E. KASHTAN, RYOCHI NEMORI, RAKESH K. SINGH, DANIEL T. MEEHAN, KATHYRN ROGERS, BRIAN R. BERRIDGE, GAUTAM BHATTACHARYA, and DOMINIC COSGROVE

Boys Town National Research Hospital, Omaha, Nebraska; Texas A & M University, College Station, Texas; University of Minnesota Medical School, Minneapolis, Minnesota; Fuji Photo Film, Co., Ltd., Kanagawa, Japan; and University of Nebraska Medical Center, Omaha, Nebraska

Increased expression of MMP-2, MMP-9 (type IV collagenases/gelatinases), and MT1-MMP in canine X-linked Alport syndrome (XLAS).

Background. Alport syndrome is a group of genetic disorders resulting from mutations in either the α3(IV), α4(IV) or α5(IV) collagen chains. The disease is characterized by a progressive glomerulonephritis, usually associated with a high-frequency specific sensorineural hearing loss, dot and fleck retinopathy, and lens abnormalities. Dogs with naturally occurring genetic disorders of basement membrane collagen (type IV) may serve as animal models of Alport syndrome. In this study, a well-characterized naturally occurring canine model was employed to demonstrate a potential role for matrix metalloproteinases (MMPs) in Alport renal disease pathogenesis.

Methods. Adolescent male dogs that developed renal failure were euthanized and necropsied. Clinicopathologic features of the disease were characterized, and kidneys from normal and Alport dogs were analyzed by gelatin zymography, Western blotting, in situ zymography, immunohistology, and by reverse transcription polymerase chain reaction (RT-PCR) for expression of MMP-2, MMP-9, and membrane type 1-MMP (MT1-MMP).

Results. Affected dogs developed proteinuria and rapidly progressive juvenile-onset chronic renal failure. The activities of MMP-2 and MMP-9 were significantly induced in Alport kidneys. In situ zymography confirmed elevated active metalloproteinases in kidney cryosections of affected dogs. The mRNAs encoding MMP-2, MMP-9, and MT1-MMP were also increased in Alport dogs suggesting that elevated expression of MMPs reflects events in the progression of Alport syndrome in dogs.

Conclusion. Elevated expression of MMP-2, MMP-9, and MT1-MMP is observed in fibrotic renal cortex from X-linked Alport syndrome dogs. These findings suggest that MMPs may play an important role in matrix accumulation associated with progressive renal scarring in this model.

Key words: Alport syndrome, fibrosis, matrix metalloproteinase.

Alport syndrome (AS) is a group of genetic disorders resulting from mutations in collagen IV genes. The pathologic hallmark of the disease is irregular thickening, thinning, and splitting of the glomerular basement membrane (GBM) revealed by electron microscopic examination of renal tissue. Patients with AS frequently have progressive high-frequency sensorineural hearing loss, retinal flecks, and lens abnormalities in addition to the renal phenotype [1]. In humans, most cases of AS are caused by mutations in the collagen α5(IV) gene, which resides on the X chromosome, and thus results in a severe dominant form of the disease in males, and a less severe phenotype in females due to random X chromosome inactivation resulting in phenotypic mosaicism [2, 3]. Autosomal-recessive forms of AS are less common (about 15% of cases), and result from mutations in the collagen α3(IV) and α4(IV) genes [4, 5]. In human AS, the mutations in α3(IV), α4(IV), or α5(IV) chains of type IV collagen are often associated with a loss of these chains from the GBM, leaving only collagen α1(IV) and α2(IV) chains, although exceptions have been noted [6]. Two gene knockout mouse models and a random transgene insertion mouse model for autosomal Alport syndrome have been described [7–9]. Three different dog models, including two with X-linked inheritance and one with autosomal inheritance, have been described [10–13]. All of these models are deficient in collagen α3(IV), α4(IV), and α5(IV) chain deposition in the GBMs, and all culminate in glomerular and interstitial fibrosis.

Clinicopathologic studies of various glomerular diseases have demonstrated that tubulointerstitial pathology correlates with the degree and progression of renal impairment, regardless of the type and anatomic origin of the inciting injury [14, 15]. The interstitial matrix deposited in the scarred kidney is produced by resident
cells, including activated fibroblasts and tubular epithelial cells with a fibrinogenic phenotype [16]. A net accumulation of matrix may result from changes in synthesis, degradation, or both. Changes in synthesis of matrix components in fibrosis have been addressed; however, the contributing role of matrix turnover has not received similar attention.

Developmental and homeostatic remodeling of the extracellular matrix (ECM) is a highly regulated process orchestrated by a family of zinc-containing, calcium-dependent, secreted neutral proteases known as the matrix metalloproteinases (MMPs). This family of enzymes, which now contains over 25 members, can collectively degrade all structural proteins of the ECM, including interfascial collagens (I, II, III and V), basement membrane collagens (IV), fibronectin, laminin, proteoglycans and elastin. The MMPs include collagenases, gelatinases, stromelysins, macrophage metalloelastase, matrilysin, and membrane-type MMPs. They are regulated at the level of gene transcription by latent proenzyme activation and are inhibited by a group of inhibitor proteins known as tissue inhibitors of MMP, which consist of four family members (TIMP-1 to TIMP-4) [17, 18].

The relative balance of MMPs and TIMPs is thought to determine the rate of ECM turnover. An imbalance in these ratios can contribute to the progression of some pathologic disorders, including tumor invasion and metastasis, arthritis, fibrotic diseases, atherosclerosis, and emphysema [19]. A highly regulated balance of active MMPs and TIMPs is maintained during normal tissue metabolism. To date, there have been no in vivo studies of the contribution of MMPs and TIMPs to the development of fibrosis in AS. Using dogs with X-linked Alport syndrome, we studied the expression of MMP genes and histologic evidence of kidney fibrosis. The results of our study revealed a strong correlation between MMP gene expression, MMP gelatinase activity, and histopathologic evidence of fibrosis, suggesting that deregulated matrix remodeling may contribute to the pathology of the disease.

METHODS

Dogs

Animals were from a colony of dogs originally referred to as the Navasota kindred, which is on a mixed background [11]. Recently, a 10 base pair deletion has been identified in exon 9 of the type IV collagen α5 gene, confirming these dogs serve as a genetic model for XLAS (Cox et al, unpublished). Progression of renal disease in Alport dogs was monitored to a standardized end point, serum creatinine ≥5.0 mg/dL (normal reference range, 0.5 to 1.5 mg/dL) or onset of uremic symptoms (anorexia and vomiting on 2 consecutive days), whereupon each dog was necropsied. Normal dogs used as controls were unaffected littermates of Alport dogs. Renal fibrosis scores were based on a semiquantitative assessment of the extent of cortical interstitial fibrosis observed in 3 μm thick, hematoxylin and eosin–stained sections of kidney from each dog. Sections were assigned scores on a scale from 0 to 3+, with 0 representing normal kidney with scant to no interstitial connective tissue, and 1+ to 3+ representing progressively increasing severity of interstitial fibrosis. Fibrosis scores of 3+ were representative of the upper limit of severity of renal cortical fibrosis generally observed in male Alport dogs necropsied at the standardized end point used in this study. For our studies, necropsy specimens of kidney from five Alport dogs and five normal dogs were analyzed.

Tissue processing

Necropsies were performed after euthanasia and a portion of renal cortex was formalin-fixed and later paraffin-embedded, sectioned, and stained for light microscopic examination. Another portion was immediately immersed in liquid nitrogen and kept at −80°C until use. For further processing, tissues were thawed, weighed, and homogenized in Tris buffer (0.5 mol/L Tris-HCl, pH 7.5, containing 200 mmol/L NaCl and 0.2% Triton X-100), centrifuged, and the supernatants were aliquoted and stored at −80°C. The protein content in the supernatants was determined using the micro BCA assay kit according to the manufacturer’s instructions (Pierce Biochemicals, Rockford, IL, USA) and stored at −20°C until used.

Gelatin zymography

Substrate gel electrophoresis (zymography) was performed to identify whether the extracts contained MMP activity and to identify the enzymes involved. The gelatin-degrading activity was examined by electrophoresis on an 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing gelatin (1.0 mg/mL) without prior heating or reduction as described [20, 21]. Prestained SDS-PAGE protein standards (Bio-Rad Laboratories, Richmond, VA, USA) and conditioned media from human HT1080 cells, which contain MMP-2 and MMP-9, were run in parallel to determine the molecular weights of gelatinases present in kidney tissue extracts. After electrophoresis, the gels were washed 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. Gels were stained with Coomassie Brilliant Blue R250 and destained. Gelatinolytic activity of each gelatinase is evident as a clear band against the blue background of stained gelatin. The addition of molecular weight markers and MMP-2 standard to the gels facilitated identification of the enzymes. 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. This confirmed the linearity of zymography (not shown).
In order to determine whether clarified zones were due to MMPs or to serine or aspartic proteases, duplicate gels were incubated in proteolysis buffer with addition of appropriate inhibitors for the enzyme class. The inhibitors used were 10 mmol/L ethylenediaminetetraacetic acid (EDTA) for MMPs; 1.0 mmol/L phenylmethylsulfonyl fluoride (PMSF) for serine proteases, and 5.0 μmol/L pepstatin A for acid and aspartic proteases.

Detection of gelatinolytic activity by film in situ zymography (FIZ)

The gelatinolytic activity was also detected by FIZ using cross-linked gelatin film (gelatinolytic-type film) according to manufacturer’s instructions (Fuji Photo Film Co., Ltd., Tokyo, Japan) with modifications. Briefly, samples of kidney were embedded without fixation in Tissue-Tek 22-oxacalcitriol (OTC) compound (Sonkura Fine-tek, Torrance, CA, USA). These frozen blocks were then sliced sequentially using a cryostat microtome to prepare serial frozen-thin sections (6 μm thickness). These thin sections were placed on a polyethylene terephthalate base film (Fuji Photo Film Co., Ltd.), coated with cross-linked gelatin of 7 μm in thickness. The films with sections were incubated for 12 hours at 37°C in a humidified chamber and stained with 1% Amido Black 10B in 70% methyl alcohol, 10% acetic acid, and 10% glycerol for 10 minutes, destained in 70% methyl alcohol, 10% acetic acid, and 10% glycerol for 10 minutes and kept in 20% glycerol for 20 minutes. The sections were cover-slipped, sealed, and imaged. The gelatin in contact with the proteolytic areas of the sections was digested, and thus negative staining indicated zones of enzymatic activity. To confirm the specificity of in situ zymography, frozen sections were also placed on gelatin-coated films containing 1,10-phenanthroline (gelatinase inhibitor [GI] films), which is known to inhibit the activity of MMPs.

Immunofluorescence evaluations

Kidneys were embedded in Tissue-Tek OTC aqueous compound. They were frozen and sectioned at 4 μm on a cryostat. Tissue sections were fixed for 10 minutes in acetone. After washing the slides in phosphate-buffered saline (PBS), they were incubated in a moist chamber with the appropriate dilution of primary antibodies. The antibodies used were anti-human MMP-2, MMP-9 (a gift from Dr. Z. Gunza-Smith, Miami, FL), and rabbit polyclonal anti-MT1-MMP antibodies (Calbiochem, La Jolla, CA, USA). After washing, the slides were incubated with affinity-purified fluorescein isothiocyanate (FITC)-conjugated goat antirabbit immunoglobulin G (IgG) (Vector Laboratories, Burlingame, MA) and mounted with Vectashield mounting media for fluorescence (Vector Laboratories). The sections were cover-slipped, sealed, and imaged. Images were collected using an Olympus (Tokyo, Japan) BH-2 fluorescence microscope with Plan APO lenses configured with a SpotRT digital camera and Image Pro-plus software (Tokyo, Japan). Tissue sections were reacted with preimmune serum and then with secondary antibody as a control for background immunostaining.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Semiquantitative analysis of MMP-2, MMP-9, and MT1-MMP transcripts was performed by RT-PCR as described previously [21]. Briefly, total RNA was isolated using Trizol (GIBCO BRL, Gaithersberg, MD, USA) according to the manufacturer’s instructions. Total RNA (5 μg) was reverse transcribed by using Superscript II with oligo dT primers (GIBCO BRL, Gaithersberg, MD, USA).

The reverse transcribed cDNA was used in each PCR, in a total volume of 50 μL, with specific primers for target molecules [MMP-2, MMP-9, MT1-MMP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]. Semiquantitative of MMP-2, MMP-9, and MT1-MMP cDNAs was performed using densitometry and comparison to GAPDH (internal standard) from the same sample after using an image analysis system (Imagequant Software, Molecular Dynamics, Sunnyvale, CA, USA). The intensity of each band was expressed in arbitrary units.

The oligonucleotide primer pairs used were as follows: canine MMP-2 (GeneBank accession no. AF177217), 5'-GCC CCA AGA AGA GAT GCA ACC TAT-3' (sense) and 5'-CGG CCA AAG TTG ATC ATG TC-3' (antisense); human MMP-9 (GeneBank accession no. J05070), 5'-GCT CCC CCC ACT GCT GGC CCT TCT ACG GGC-3' (sense) and 5'-GTC CTC AGG GCA CTG CAG GAT GTA ATA GAT-3' (antisense); canine MT1-MMP (GeneBank accession no. AF097638), 5'-GTT TTG ATG AGG CTT CTC TGG A-3' (sense) and 5'-TCC AGT ATT TGT GCC CCT TGT A-3' (antisense); and human GAPDH (Genebank accession no. M33197), 5'-GGT GAA GGT CCG AGG AGT CAA CGG ATT TGG TCG-3' (sense) and 5'-GGA TCT CGC TCC TGG AAG ATG GTG ATG GG-3' (antisense). Each PCR was carried out at various numbers of cycles under identical conditions to optimize the reactions. The amplified products were identified by electrophoresis of an aliquot on a 2% agarose gel stained with ethidium bromide, visualized by ultraviolet transillumination, and photographed. DNA bands were analyzed by computer-assisted densitometric scanning of these images using an image analysis system (Imagequant Software, Molecular Dynamics). The intensity of each amplified product increased with the increasing number of PCR cycles. The number of PCR cycles for each product was determined to lie in the linear range with respect to specific band intensity. The PCR cycling parameters for MMP-2 were as follows: initial denaturation temperature at 94°C for 10 minutes
Fig. 1. General morphology of renal cortex from X-linked Alport syndrome (XLAS) dogs typical of those used in this study. Tissue was embedded in paraffin, cut at 3 μm, and stained using hematoxylin and eosin. Fibrosis scores, determined as described in the text, are indicated. C is normal.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Status</th>
<th>Gender</th>
<th>Age at necropsy months</th>
<th>Serum creatinine mg/dL</th>
<th>Renal fibrosis score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>Male</td>
<td>12</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>Male</td>
<td>7</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>Male</td>
<td>7</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Normal</td>
<td>Male</td>
<td>11</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Normal</td>
<td>Male</td>
<td>11</td>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Affected</td>
<td>Male</td>
<td>11</td>
<td>5.0</td>
<td>2+</td>
</tr>
<tr>
<td>7</td>
<td>Affected</td>
<td>Male</td>
<td>13</td>
<td>5.5</td>
<td>2+</td>
</tr>
<tr>
<td>8</td>
<td>Affected</td>
<td>Male</td>
<td>8</td>
<td>5.0</td>
<td>3+</td>
</tr>
<tr>
<td>9</td>
<td>Affected</td>
<td>Male</td>
<td>7</td>
<td>4.9</td>
<td>3+</td>
</tr>
<tr>
<td>10</td>
<td>Affected</td>
<td>Male</td>
<td>11</td>
<td>5.1</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 1. Animals used in the study

Specific cycle numbers were as follows: for MMP-9, 30 cycles; for MT1-MMP, 34 cycles; and GAPDH, 25 cycles. In each instance, the constitutively expressed GAPDH mRNA was used as an internal standard for RT-PCR. The PCR procedure was repeated at least three times for each sample. No PCR product was observed in samples in which either cDNA or reverse transcriptase was omitted. To control for sample variation due to handling, band intensities for each unknown were normalized to band intensities for GAPDH, which was amplified in parallel.

DNA sequencing

The MMP-2, MMP-9, MT1-MMP, and GAPDH fragments amplified from XLAS and control kidneys by PCR were purified using Multiscreen PCR. The resultant DNA was subjected to a cycle sequencing reaction using the ABI Prism dye-labeled terminator reagent (PE Biosystems, Foster City, CA, USA), according to the protocol provided by the manufacturer. The reaction product
was analyzed with an ABI automated DNA sequencer, model 377 (Applied Biosystems, Palo Alto, CA, USA).

**Western blot analysis**

The specificity of MMP-2, MMP-9, and MT1-MMP antibodies used in the immunohistochemical analyses was characterized by Western blot analysis. The tissue extracts were subjected to standard 8% SDS-PAGE under reducing conditions as described earlier [21]. Proteins were then electrophoretically transferred onto a nitrocellulose membrane, blocked with 5% nonfat dry milk in Tris-buffered saline plus 1% Tween-20 (TBST) (Bio-Rad, Hercules, CA, USA), followed by incubation with diluted rabbit polyclonal antibodies (MMP-2, 1:300; MMP-9, 1:300; and MT1-MMP, 1:500) overnight at 4°C. The membranes were washed again three times with TBST and were incubated with peroxidase-conjugated secondary rabbit antibody. Finally, the color was developed using diaminobenzidine and hydrogen peroxide as described [22].

**Data presentation and statistical analysis**

Data are expressed as mean ± SD. Differences between means were tested for significance using Student t test. Differences were considered significant at the level of $P < 0.05$.

**RESULTS**

**Clinicopathologic evaluations**

The Alport dogs manifested progressive renal disease typical of that previously described in males from this
Affected dogs used in this study developed persistent proteinuria [urine protein to creatinine (UPC) ratio > 0.5 then and thereafter] at 3 to 5 months of age. Onset of azotemia (serum creatinine ≥ 1.6 mg/dL) occurred at 4 to 8 months of age, and their renal failure progressed to the standardized end point at 7 to 13 months of age. Normal dogs did not exhibit proteinuria (UPC ≤ 0.5) and were not azotemic. Interstitial fibrosis was not observed in the kidneys of normal dogs. However, the tubulointerstitium of the renal cortex of all Alport dogs was expanded by fibrous connective tissue admixed with mixed mononuclear inflammatory cells typical of the chronic inflammatory renal changes associated with this syndrome. Characteristic histology of renal cortex from normal dogs and from Alport dogs that were scored at 1+, 2+, and 3+ are provided in Figure 1. Ages at necropsy, serum creatinine levels, and renal fibrosis scores of dogs used in this study are summarized in Table 1.

We examined MMP-2, MMP-9, and MT1-MMP expression in normal and X-linked Alport syndrome (XLAS) renal cortex. Representative fields from normal and XLAS dogs with a fibrosis score of 2 are shown in Figure 2. No obvious qualitative differences were observed when comparing sections from animals with a fibrosis score of 2 or 3. Expression of MMP-2 and MMP-9 in controls is weakly evident in the tubular epithelial cells (Fig. 2A and B). In Alport dogs, however, brightly stained cells are evident in the interstitial space. MMP-9 also appears to accumulate in the basement membranes and the interstitial matrix near clusters of brightly stained cells (Fig. 2E). MT1-MMP expression is again very low in the tubular epithelial cells in normal dog kidneys; however, in XLAS kidneys, elevated immunostaining is observed at the interface between the tubular epithelial cells and the tubular basement membranes. This cell surface localization is consistent with the fact that MT1-MMP is a membrane bound proteolytic activator for MMP-2 [23].

Since none of these antibody preparations have previously been used to detect MMPs in dog tissues, it necessary to evaluate their specificity. To test for this, tissue extracts from a control and Alport dogs were subjected to Western blot analysis. The blots developed with the antibodies demonstrated a single band for each antibody with the predicted molecular size (Fig. 3). This confirms that the antibodies are indeed specific, and cross-reactive with the respective dog MMPs.

Detection of MMP activity by gelatin zymography

Proteolytic activity of two forms of type IV collagenase (72 kD, MMP-2; 92 kD, MMP-9) in control and XLAS dog kidney extracts from necropsy samples was analyzed by gelatin zymography. Figure 4 is provided to validate the specificity of the assay to detect latent and active forms of the MMPs. Extracts from XLAS kidney contained several protease activities at molecular weights of 92, 84, 72, and 64 kD. The upper gelatinolytic bands represent the 92 kD (latent or proform) and 88 kD (active form) MMP-9 species, whereas the lower gelatinolytic bands represent the 72 kD (latent or proform) and 64 kD (active form) MMP-2 species. Standards, which are supernatants from human fibrosarcoma (HT1080) cells, are predominantly the latent forms of MMP-2 and MMP-9.

The gelatinase activity was completely inhibited by EDTA, a chelating agent, confirming that the observed bands were the result of zinc-dependent metalloproteinase activity. The gelatinase bands on the zymogram were not inhibited by PMSF, an inhibitor of serine proteases, or pepstatin A, an inhibitor of aspartic proteases.

Analysis of gelatinase activity in the renal cortex of XLAS dogs

Gelatin zymography was performed using extracts of renal cortex from control and XLAS dogs. Extracts were derived from necropsy specimens taken from individuals with either moderate fibrosis score (2+) or severe fibrosis score (3+). The results in Figure 5 illustrate that significant differences in the levels of MMP-2 and MMP-9 were evident in the XLAS necropsy kidney extracts when compared to normal dogs. The active forms of both MMP-2

![Fig. 3. Western blot analysis of matrix metalloproteinases (MMP-2 and MMP-9) and membrane type 1 MMP (MT1-MMP) in normal and X-linked Alport syndrome (XLAS) tissue extracts.](image-url)
Rao et al: MMPs in canine Alport syndrome

Fig. 4. Specificity of gelatin zymography for detecting matrix metalloproteinase (MMP) activity in tissue extracts. Lane 1, media from human fibrosarcoma cells (positive control); lane 2, tissue extract from unaffected; and lane 3 from an X-linked Alport syndrome (XLAS) dog with a fibrosis score of 3+. Both latent and active forms of MMP-2 and MMP-9 are denoted. Labels below the lanes indicate what was added to the gel incubation buffer, illustrating that the gelatinase activity is inhibited by ethylenediaminetetraacetic acid (EDTA), which is an inhibitor of MMPs, but not by phenylmethylsulfonyl fluoride (PMSF) or pepstatin A, demonstrating that activity is not due to serine or aspartate proteases.

Fig. 5. Interstitial disease in the X-linked Alport syndrome (XLAS) dogs is associated with markedly elevated matrix metalloproteinases (MMP-2 and MMP-9) activity. Sample numbers are reflecting animals described in Table 1. (A) Gelatin zymography for MMP-2 and MMP-9 proteolytic activity in necropsy tissue extracts from normal versus XLAS dogs. Quantitative analysis of MMPs. Gelatin zymograms were analyzed by scanning densitometry and expressed as arbitrary units to produce histograms. (B) The activity of total MMP-2 and MMP-9 are elevated in XLAS dogs compared to unaffected dogs. Total gelatinolytic activity represents the sum of both MMP-2 and MMP-9 activities. (C) Latent and active MMP-9 for individual samples are shown. (D) The values for total, MMP-2, and MMP-9 (both latent and active MMP-9 are shown) as mean and standard deviation for control and Alport dogs. The zymogram shown is representative of three independent experiments. *Statistical significance compared with control kidney extracts at $P < 0.001$. 
and MMP-9 were observed only in XLAS tissue extracts (Fig. 5A). Zymographic bands were quantified by scanning densitometry and the data analyzed using Image Quant software (see Methods section) and expressed as arbitrary units of lysis. There was a significant increase in the levels of both MMP-2 and MMP-9 in XLAS dogs in comparison to controls (P < 0.001) (Fig. 5B). Fold induction was remarkably high, especially for MMP-9, and did not vary substantially in samples derived from animals with fibrosis scores of 3+ compared to 2+ (an average of 89-fold versus 72-fold, respectively, for MMP-9, and 7.5-fold versus 6.6-fold, respectively, for MMP-2, see Table 2). Active MMP-9 is seen only in affected animals and not in controls (Fig. 5C).

In situ zymography for the detection of gelatinase activity by FIZ

In situ zymography using gelatin films (gelatinolytic films) on cryosections revealed that strong gelatinolytic activity is present in specimens from XLAS dogs, but very little or no activity was recognized in the normal dogs (Fig. 6). The region where gelatinolysis occurred was observed as white to pale blue. The gelatinolytic activity in the cryosections was almost completely inhibited on sections that have been incubated on the film containing 1,10-phenanthroline (GI film), a broad MMP inhibitor. The FIZ gelatin films employed in this assay have been shown to detect only the active isomers of the MMPs [24], confirming that a marked increase in overall metalloproteinase activity is evident in the Alport renal cortex. Furthermore, this activity appears predominantly in the interstitial space, and not in the glomeruli (denoted as G in Fig. 6).

Detection of mRNA for MMP-2, MMP-9, and MT1-MMP

RT-PCR analysis was used to determine whether the induction of MMP-2 and MMP-9 protein was secondary to the increased expression of MMP-2 and MMP-9 mRNA transcripts in XLAS kidneys. RNA was isolated from a portion of the same necropsy specimen employed in the gelatin zymography experiments. Corresponding sample numbers were derived from the same animals employed for gelatin zymograms (Fig. 5). The primers selected for amplification produced only a single product of the predicted size for selected cDNA fragments encoding the MMPs and GAPDH, a housekeeping gene that was used to control for cDNA loading in the PCR reactions. Sequencing of amplified cDNAs from control and XLAS indicated 100% homology with the published sequences validating that the amplified products were indeed specific (not shown). The most dramatic induction of MMP mRNA expression in XLAS was that for MMP-9 (Fig. 7A, middle panel), consistent with the data obtained through gelatin zymography. RT-PCR amplification of MMP-9 produced a cDNA of the expected size, which was barely detectable in controls but was significantly increased (19-fold on average, see Table 2) in XLAS (P < 0.001). The relatively low levels of MMP-9 amplification product in controls was not the result of the quality or quantity of mRNA because the presence of intact mRNA, as well as equivalent loading of reverse transcribed message in the PCR reactions was demonstrated by amplification of GAPDH mRNA (Fig. 7A, lower panel). Amplification of MMP-2 (Fig. 7A, upper panel) confirms expression in both control and XLAS dogs and elevated expression (twofold on average, see Table 2) in XLAS dogs as compared to control (P < 0.001). The relative expression of MMP-2 and MMP-9 specific mRNA transcripts in XLAS as determined by densitometric analysis of RT-PCR is given in Figure 7C. These results were qualitatively in agreement with zymography (gelatin and in situ) and immunofluorescence analysis.

Fold induction of mRNA versus gelatinolytic activity, however, revealed some interesting similarities. Fold induction of MMP-2 and MMP-9 gelatinolytic activity was greater than that for MMP-2 and MMP-9 mRNA. The ratio for fold induction (in XLAS versus control samples) of enzyme activity to mRNA, on average, is between 3 and 4 to 1 for both MMP-2 and MMP-9. Gelatinolytic activity and specific mRNA levels for the MMPs were not significantly different for samples taken from animals with a fibrosis score of 2+ compared to 3+.

Transcripts for MT1-MMP mRNA were also analyzed by RT-PCR and found to be consistently and significantly elevated (2.6-fold on average, Figure 8 and Table 1) in XLAS-derived samples relative to controls (P < 0.001). These data reflect elevated MT1-MMP protein observed by immunofluorescence (Fig. 2 C and F).

### Table 2. Summary of changes in matrix metalloproteinase (MMP) activity and mRNA levels relative to that observed in normal dogs

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Serum creatinine mg/dL</th>
<th>Fibrosis score</th>
<th>MMP activity</th>
<th>mRNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MMP-2</td>
<td>MMP-9</td>
</tr>
<tr>
<td>6</td>
<td>5.4</td>
<td>2+</td>
<td>6.8 ± 0.5</td>
<td>75.3 ± 8.2</td>
</tr>
<tr>
<td>7</td>
<td>5.0</td>
<td>2+</td>
<td>6.5 ± 0.7</td>
<td>68.7 ± 7.5</td>
</tr>
<tr>
<td>8</td>
<td>4.9</td>
<td>3+</td>
<td>7.4 ± 0.8</td>
<td>87.2 ± 8.3</td>
</tr>
<tr>
<td>9</td>
<td>5.3</td>
<td>3+</td>
<td>7.4 ± 0.8</td>
<td>82.5 ± 9.1</td>
</tr>
<tr>
<td>10</td>
<td>5.2</td>
<td>3+</td>
<td>7.6 ± 0.8</td>
<td>98.6 ± 10.1</td>
</tr>
</tbody>
</table>

Data are expressed as the mean of three independent measurements with standard deviations. MTI-MMP is membrane type 1 matrix metalloproteinase.
Rao et al: MMPs in canine Alport syndrome

Components, including interstitial and basement membrane collagen and glycoproteins and play an important role in various physiologic and pathologic conditions [25, 26]. Here we show that the MMP-2, MMP-9, and the membrane-bound proteolytic activator for the MMPs, MT1-MMP, are all up-regulated in the renal cortex of XLAS dogs. Induction is apparent at both the level of mRNA and enzyme activities. Further, in situ zymography reveals the presence of active metalloproteinase in tissue sections of XLAS renal cortex, demonstrating that the elevated expression translates to elevated metalloproteinase activity in the organ. Combined, these data provide compelling evidence suggesting a role for MMPs in aberrant matrix remodeling associated with interstitial fibrosis in the canine XLAS model. An earlier study examined the role of MMP-9 in glomerular pathogenesis in a murine Alport model and concluded that it did not influence disease progression. This study, however, did not address tubulointerstitial disease [27].

The regulation of kidney ECM remodeling during normal tissue metabolism and its dysregulation in kidney diseases may result from a complex interplay of MMP transcriptional regulation, proenzyme activation, and inhibition by TIMPs. In interstitial fibrosis, the primary insult initiates a sequence of events starting with the influx of a variety of inflammatory cells, including monocytes. These cells produce a variety of cytokines, growth factors and other mediators that presumably function to repair the damaged tissue and modulate expression of the MMPs [28]. Some MMPs, including MMP-2, MMP-3, MMP-9, and metalloelastase, are potentially capable of disrupting the kidney architecture by virtue of their specificity for various components of basement membrane, thus enabling further infiltration of inflammatory cells into the kidney and thereby setting into motion the dysregulated matrix remodeling that follows. Recently, we have demonstrated significantly elevated levels of MMP-2 and MMP-9 in a mouse model for AS, which progressively develops interstitial fibrosis between 4 and 8 weeks after birth. Further studies also demonstrated that the tissue monocytes, independent of myofibroblasts, drive tubular atrophy in the Alport mice [29]. The data presented here are the first to demonstrate differential expression of gelatinases and MT1-MMP genes in canine XLAS, an animal model for human XLAS. Our results suggest that differential MMP expression may contribute to the molecular pathogenesis of the disease.

We show that XLAS extracts contained activated forms of MMP-2 and MMP-9 but the mechanism for the activation is not clear. MMPs are secreted as inactive latent precursors (zymogens) and their activation is a prerequisite to their functioning in vivo [25, 26]. Activation of MMP-2 is not mediated by serine proteases, such as plasmin, as is the case for other MMPs. Instead, protease inhibitor studies suggest that MMP-2 is activated by met-
alloproteinases themselves and requires interaction with cell surface proteins [30, 31]. This unique property of MMP-2 activation likely directs proteolytic activity to either migrating cells or cells involved in ECM turnover. Recently, the membrane-bound MT1-MMP has been identified as a potential physiologic activator of MMP-2 on the cell surface. Studies have shown that a complex of MT1-MMP mediates activation of proMMP-2 with TIMP-2, acting as a surface receptor for proMMP-2 [32–34]. In cell culture, MT1-MMP expression can be stimulated by the lectin, concanavalin A [23], suggesting that clustering of cell surface molecules can lead to increased expression of active MMP-2. In the present study, we analyzed the expression of mRNA transcripts for MT1-MMP and found them to be significantly elevated in XLAS dogs compared to controls. The balance between TIMP-2 and MT1-MMP may be critical for determining the activation status of MMP-2. Our studies show that the active form of MMP-2, while abundant in XLAS renal cortex, is absent in controls. The absence of active MMP-2 in controls may be explained by relatively decreased activation of MT1-MMP, due to incomplete
processing of pro-MT1-MMP to its active form, instability of the MT1-MMP protein, or lack of translation of MT1-MMP mRNA into protein. It has been shown that proteolytic cleavage of pro-MT1-MMP is a prerequisite for pro-MMP-2 activation [35, 36].

A large number of proteases and organomercurials have been shown to activate other MMPs in vitro; however, the mechanisms of activation are not well documented in vivo. A potentially important mechanism for activation of latent MMPs is intermolecular activation. Both MMP-3 and MMP-10 are good activators of MMP-7 [37, 38]. MMP-3 can fully cleave pro-MMP-7 to generate an active species [37]. MMP-7 has been shown to activate human MMP-1, MMP-2, and MMP-9 [37, 39, 40]. It has been reported that MT1-MMP can activate collagenase 3 (MMP-13) and MMP-9 [41]. In addition, pro-MMP-9 can be activated by gelatinase A (MMP-2) [42], MMP-3 [43], MMP-13 [44], plasmin [45], and tissue kallikrein [46].

In the present study we show that MMP-9 activity was significantly increased in XLAS. Of the total detected MMP-9, about 35% represented active MMP-9, suggesting that the activation of MMP-9 in XLAS is significantly higher than active MMP-2. Recent studies have shown that the mRNA transcripts for MMP-3 and MMP-7 were significantly increased in XLAS kidneys (Rao and Cosgrove, unpublished). Therefore, it appears that MT1-MMP, MMP-7, and MMP-3 likely participates in MMP-2 and MMP-9 activation in this model. The coexpression of MMP-2, MMP-3, MMP-7, and MMP-9 may function as powerful machinery for ECM degradation in XLAS kidneys.

The common pathway of progressive tubulointerstitial fibrosis is the end result for most acute and progressive renal disease. The process of interstitial scarring and cellular destruction associated with interstitial fibrosis shares many characteristics from one organ to another.
Fibrosis is usually associated with a general inflammatory response that involves T cells, B cells, and monocytes/macrophages. In a mouse model for AS, we have demonstrated that tissue monocytes, independent of myofibroblasts, drive renal tubular atrophy [29]. Monocytes/macrophages have been implicated in connective tissue destruction mediated through MMPs and secrete a number of MMPs in response to various stimulants [47–49]. Therefore, it appears that monocytes may be involved in the upregulation of MMPs in XLAS kidneys. It is known that cytokines play a crucial role in the induction and perpetuation of inflammatory response [50], while MMPs are considered to be key enzymes in ECM turnover and physiologic mediators of cell migration through various barriers [51, 52].

MMPs were notably absent in the glomeruli of Alport dogs based on our immunostaining results (Fig. 2). This may reflect the differences in the fibrogenic processes of the interstitium and the glomerulus. In the interstitium, the interactive roles of monocytes and myofibroblasts are thought to collectively drive fibrosis [29]. In the Alport glomerulus, however, mesangial expansion associated with matrix deposition in the GBMs precedes overt glomerular sclerosis [7, 53].

CONCLUSION
This is the first report showing differences in MMP-2, MMP-9, and MT1-MMP expression in canine XLAS kidneys. Our study demonstrated significant alterations of MMP expression in canine XLAS kidneys. Elevated MMPs may serve as a compensatory mechanism aimed at limiting the rate of fibrogenesis. Fibrosis proceeds, however, despite the elevated MMP activity. The diverse functional properties of the MMPs warn that this view may be oversimplified. Thus, the specific role of elevated MMP activity in dysregulated matrix deposition associated with renal fibrosis remains unclear and will require further study.

ACKNOWLEDGMENTS
We thank Ms. Nicole Pearsall and Q. Kong for their help in preparing the histograms and statistical analysis, and Rene Rodgers for sequence analysis. We also thank John (Skip) Kennedy for help in figure preparation. This work is supported in part by NIH R01 DK55000 to D.C., NIH R01 DK57676 to C.E.K., and the tobacco settlement fund from the State of Nebraska.

Reprint requests to Dominic Cosgrove, Ph.D., Boys Town National Research Hospital, 533 No. 30th St., Omaha, NE 68131.

E-mail: cosgrove@boystown.org

REFERENCES


