Matrix metalloproteinases and mesangial remodeling in light chain–related glomerular damage

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Background. Matrix metalloproteinases (MMPs) belong to the zinc endopeptidase subgroup of the metalloproteinase superfamily and are primarily involved in extracellular matrix (ECM) remodeling. Alterations of the mesangial ECM in AL-amyloidosis (AL-Am) and light chain deposition disease (LCDD) are crucial in their pathogeneses as two divergent entities.

Methods. Protein expression patterns of five MMPs (MMP-1, 2, 3, 7, and 9) in renal tissues obtained from autopsies and kidney biopsies, and cultured human mesangial cells (HMCs) treated with light chains obtained from the urines of patients with AL-Am and LCDD were analyzed. MMP mRNA expressions were determined in glomeruli following laser capture microdissection and selective MMP microarray. Zymography was used to assess MMP activity.

Results. The average glomerular MMP expression was 6 times greater in AL-Am than LCDD and negative control renal tissues with different expression profiles: MMP-1 > 9 > 3 > 2, MMP-1 > 2, 9 > 3 > 7, and MMP-2, 3, 7 > 9 > 1, respectively. Microdissected glomeruli and HMCs treated with light chains expressed higher levels of MMP mRNA and proteins in AL-Am than LCDD. Zymography was used to assess activity demonstrating increased MMP-2 in AL-Am.

Conclusion. Altered expressions of MMPs play a key role in the pathogenesis of AL-Am and LCDD. MMPs were more highly expressed in AL-Am compared to LCDD.

Matrix metalloproteinases (MMPs) are structurally and functionally related zinc endopeptidases belonging to a subgroup of the much larger metalloproteinase superfamily and function primarily in extracellular matrix (ECM) remodeling [1–4]. MMPs are ubiquitous enzymes and more than 20 vertebrate types have been identified to date [5]. Eight distinct structural classes of MMPs have been designated; five classes are secreted while three are membrane tethered [6].

All MMPs are synthesized as zymogens and are secreted extracellularly or transmembranely attached [7]. Their gene expressions are inducible via many mechanisms and their activities may be inhibited by endogenous specific tissue inhibitors of metalloproteinases (TIMPs), plasminogen activation inhibitor (PAI), and other non-specific protein inhibitors such as α2-macroglobulin [8].

Although reports on some roles of MMPs in the kidney have been published, most have focused on the activities of gelatinase A and B (MMP-2 and MMP-9). These highlight the biophysical characteristics of these two closely related MMPs and the fact that they primarily act on collagen IV, the main ECM component of the mesangium. Therefore, it is not surprising that some investigators have reported MMP-2 to play a key role in glomerular basement membrane (GBM) and mesangial matrix remodeling [9–11]. To date, little association between the pathogenesis of renal damage due to plasma cell dyscrasia (multiple myeloma) and MMPs have been described. A comprehensive array of MMP expressions in human mesangial cells has not been delineated.

TIMPs are natural inhibitors of MMPs whose role is paramount in their regulation. Four TIMPs have been described and they share a 40% sequence homology [12]. However, TIMP-1 and TIMP-2 are the best described [13, 14] and likely play important roles in maintaining mesangial homeostasis. Studies by Gomis-Ruth et al [15] have revealed a unique inhibitory mechanism between the MMPs and TIMP-1. The folding of the TIMP-1 polypeptide chain into a wedge-shaped molecule such that its N- and C-terminal halves form two opposing parts blocking the entire active cleft site of MMP-3 has been described.

Plasma cell dyscrasias, including their most dramatic clinical expression referred to as multiple myeloma,
are associated with the production of physicochemically abnormal light chains, the majority of which are nephrotoxic. Approximately 30% of the pathologic light chains are associated with glomerular damage (also referred to as glomerulopathic light chains), while the remaining nephrotoxic light chains are associated with tubular interstitial pathology and referred to as tubulopathic.

Renal damage has been shown to be a prominent feature of plasma cell dyscrasias resulting in renal function disturbances [16, 17]. Just over half the number of multiple myeloma patients develop renal insufficiency and failure, the second most common cause of death in these patients, infections being the most common cause [17]. The interactions between glomerulopathic light chains and mesangial cells are mediated by a yet uncharacterized cell surface receptor and data obtained in our laboratory indicate the presence of a single receptor providing binding sites for the different glomerulopathic light chains [18, 19].

Following interactions of the glomerulopathic light chains with the surface receptor and internalization in mesangial cells, a sequence of events occurs eventually leading to alterations in mesangial matrix homeostasis. Light chains in light chain disposition disease (LCDD) are degraded in the early endosomes while, in AL-amyloidosis (AL-Am) they are transported to the mature lysosomes for processing [19]. Amyloidogenic light chains undergo proteolysis in the mature lysosomal compartments leading to the formation of fibrils which are then extruded into the ECM where they accumulate and alter the mesangial milieu.

As a consequence, two distinct characteristic patterns of glomerular injury emerge. Fibrillar monoclonal light chain–related renal disease, characteristic of AL-Am, and granular deposition of immunoglobulin components typical of LCDD constitute these patterns (Fig. 1). λ light chains are approximately two to three times more commonly associated with AL-Am are than κ [20, 21]. Solomon, Frangione, and Franklin [22] reported that λVI light chains are more specifically associated with AL-Am while Comenzo et al [23] demonstrated a striking tropism of the λVI light chains for glomerular amyloidosis. Conversely, LCDD is usually associated with κI and κIV light chains. Therefore, the physicochemical characteristics of the light chains alter the behavior of mesangial cells resulting in mesangial matrix alterations [24–29].

This manuscript presents evidence of variations in MMP expressions by mesangial cells from AL-Am and

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Fig. 1. Characteristics of the AL-amyloidosis (AL-Am) and light chain disposition disease (LCDD) glomeruli. Replacement of mesangial matrix by eosinophilic amorphous material is noted in AL-Am (A) (hematoxylin and eosin ×400). Decreased extracellular matrix (ECM) is demonstrated by lack of silver staining in expanded mesangial areas (B) (Jones methanamine silver ×400), confirmation of amyloid deposits (C) (Congo red, polarized light, ×400), and (D) (Congo red, bright field ×400), and (E) amyloid fibrils (electron microscopy uranyl acetate and lead citrate ×9500). Note transformed mesangial cells with large lysosomes (macrophage phenotype) surrounding amyloid fibrils. Mesangial nodules (F to H) (hematoxylin and eosin ×400) with increased mesangial matrix protein deposition (G) (Jones methanamine silver ×400) and punctuate light chain deposits (H) (electron microscopy uranyl acetate and lead citrate ×9500) in the mesangium of LCDD glomeruli.
LCDD patients at both the mRNA and protein levels. Also, such changes were investigated in cultured human mesangial cells (HMCs) incubated with different light chains.

METHODS

Definition of terms

Myeloma cast nephropathy (MCN) results from precipitation of light chains in distal tubules resulting in cast formation. These light chains do not affect the glomerulus and serve as a light chain control.

Negative controls are used in the analyses of glomeruli and consist of (I) normal, which is tissue taken from uninvolved portions of renal carcinoma nephrectomy specimens; (2) acute tubular necrosis (ATN), which is characterized by injury resulting in the necrosis of tubular cells without glomerular alterations; and (3) postperfusion effect (PPE), which results in proximal tubular injury with no glomerular alterations, as consequence of renal perfusion aimed at organ preservation prior to transplantation.

Positive control used in the analysis of renal tissues consisted of thrombotic microangiopathy (TMA) cases. This is characterized by thrombotic occlusion of small vessels in the renal vasculature resulting in mesangiolysis and, at times, overt glomerular necrosis associated with the activation of metalloproteinases.

Isolation of HMCs

HMCs were obtained from unused kidney tissue that was earmarked for transplantation, or from normal areas of nephrectomy tissue removed for neoplastic conditions. The cortices from these kidneys were minced and pressed onto sterile stainless steel sieves of various pore diameters (315, 250, and 180 μm) as previously described[30]. Whole glomeruli retained on the 180 μm sieve were collected then washed in Hank’s buffered saline solution (HBSS) and centrifuged. The glomerular pellets were resuspended in Hepes-buffered HBSS (pH 7.4) containing 750 μg/mL collagenase IV (Sigma Chemical Co., St. Louis, MO, USA). After 30 minutes incubation with gentle agitation, the suspension was centrifuged at 1000 rpm for 10 minutes. The pellets were washed twice and resuspended in complete medium containing RPMI 1640 (Life Technologies-Gibco, Grand Island, NY, USA), buffered with 12.5 mmol/L Heps (Sigma Chemical Company), supplemented with heat inactivated 20% fetal calf serum (FCS) (Life Technologies), penicillin 100 U/mL, streptomycin 100 μg/mL, 5 μg/mL bovine insulin, 2 mmol/L glutamine, 5 μg/mL transferrin, 5 μg/mL selenite, and 1 mmol/L sodium pyruvate (all from Sigma Chemical Co.), and plated onto 100 μm² tissue culture dishes.

The glomerular cells were carefully assessed for 3 to 5 days. Once outgrowths were established, the cells were trypsinized, passed through a 75 μm sieve to remove the whole glomeruli, and replated on tissue culture dishes. The cells were maintained at 37°C in a humidified incubator containing 5% CO₂. Mesangial cells overgrew epithelial cells and became confluent 3 to 4 weeks after plating. Cells were then trypsinized and transferred to 8-well chambered slides. Ultrastructural examination and immunohistochemical staining positive for muscle-specific actin and vimentin, and negative for keratin and factor VIII, confirmed the presence of a homogeneous population of mesangial cells. These mesangial cells were then frozen at −70°C and passaged for future experiments.

Isolation and purification of light chains

Free light chains were purified from the urine of patients with known LCDD or AL-Am or MCN, used as a tubulopathic light chain control, which has been proven not to interact with mesangial cell receptors and do not alter transforming growth factor β (TGF-β) [31, 32] by a series of affinity chromatography steps. Urine was passed over an affinity column comprised of either goat anti-human κ or λ light chain antibody conjugated to sepharose CL-4B. Free and bound light chains were eluted from the column with 0.1 mol/L glycine, pH 2.2. The purity of the light chains were assessed on immunodiffusion plates and contaminating proteins (IgG and IgA) were removed by subsequent repeated passages over affinity columns to which either anti-human IgG or IgA antibodies were conjugated.

Purified light chains migrated as a single band when resolved by zone electrophoresis on agarose gels using a 50 mmol/L barbital buffer, pH 8.6, and consisted entirely of either free κ or λ protein as determined by immunofixation. The purified light chain was then concentrated, dialyzed against 0.9% NaCl, filtered through a sterile 0.2 μm membrane, and stored aseptically in sealed vials at 4°C. Purified light chains from individual patients were used for each of the experiments. Light chains were not pooled by categories for the experiments.

Incubation of HMCs with light chains

HMCs of third passage were thawed from stocks of harvested cells previously described. These cells were seeded onto 8-well tissue culture glass slides and 100² mm Petri dishes in modified RPMI 1640 culture medium. RPMI 1640 containing 12.5 mmol/L Hepes, 2 mmol/L L-glutamine and phenol red (Sigma Chemical Co.) was supplemented with heat inactivated 15% fetal bovine serum (FBS) (Pierce Biotechnology, Inc., Rockford, IL, USA), penicillin 100 U/mL, streptomycin 100 μg/mL, ITS (bovine insulin 5 μg/mL, 5 μg/mL transferrin, and 5 μg/mL selenite), and 1 mmol/L sodium pyruvate (Sigma
Chemical Co.). They were allowed to grow to approximately 90% confluence with media change every 3 days after which they were brought to quiescence for 48 hours with the same culture medium now containing 0.5% FBS. Duplicate cultures were incubated with a MCN light chain, a LCDD light chain or an AL-Am light chain diluted (10 μg/mL) in phenol red free RPMI 1640 containing 12.5 mmol/L Hepes, 2 mmol/L-glutamine, penicillin 100 U/mL, streptomycin 100 μg/mL, ITS, 1 mmol/L sodium pyruvate, and 0.5% FBS. HMCs in culture medium devoid of light chains served as control. Cells were incubated for up to 96 hours at 37°C and 5% CO2 for 96 hours. Cells and supernatants were harvested at different time intervals (12, 24, 48, 72, or 96 hours). Some cells grown in Petri dishes were harvested at 96 hours. Cells and treated with TRIzol™ Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) to obtain RNA and proteins. Conditioned media were harvested then concentrated by centrifugation using 10K concentrative devices (Pall Corporation, Ann Arbor, MI, USA) to contain 90 μg total proteins in 37 μL. Unknown samples, standards [10 μL recombinant human MMP (R&D Systems, Minneapolis, MN, USA)] and molecular marker were loaded onto NuPAGE® Novex 4% to 12% Bis-Tris (1.5 mm, 10 wells) gels (Invitrogen Life Technologies) and electrophoresed. The gels were later transferred onto polyvinylidine difluoride (PVDF) membranes by electrophoresis.

Twenty milliliters diluted anti-MMPs 1, 2, 3, 7, and 9 antibodies (R&D Systems) were diluted in a similar fashion with agitation. X-ray films were used to capture images which were later analyzed. Mean density values the protein bands were performed by densitometry on a Kodak Image Station 2000mm using Kodak Digital Science™ one-dimensional image analysis software (Rochester, NY, USA).

**Experimental controls**

Controls used in the in vitro experiments consisted of HMCs incubated without light chains in phenol red free RPMI 1640 containing 0.5% FBS. Controls in the renal tissue experiments consisted of tissue taken from uninvolved portions of renal carcinoma nephrectomy specimens, serving as negative controls, and glomeruli affected by TMA serving as positive controls. MCN-light chains were employed as an internal nonglomerulopathic light chain control to compare with the glomerulopathic light chains of interest.

**Preparation of conditioned media from in vitro experiments**

Conditioned media from the in vitro experiments were harvested then concentrated by centrifugation using 10K Macrosep® centrifugal devices (Pall Corporation, Ann Arbor, MI, USA) which were centrifuged at 2390 × g for 35 minutes at 4°C. The concentrated conditioned media were then collected in 2 mL microcentrifuge tubes and stored at −70°C until needed.

**RNA and protein isolation from HMCs using TRIzol™ Reagent**

Total RNA and proteins were isolated from cultured HMCs incubated with light chains or culture media (described above) using TRIzol™ Reagent (Invitrogen Life Technologies). RNA samples were stored at −70°C for future analysis. The protein concentrations were determined prior to Western blotting or enzyme-linked immunosorbent assay (ELISA) analyses.

**Western blot on HMC-conditioned media**

Conditioned media harvested from cultured HMCs treated with light chains were diluted in 4× lithium dodecyl sulfate (LDS) buffer (Invitrogen Life Technologies) to contain 90 μg total proteins in 37 μL. Unknown samples, standards [10 μL recombinant human MMP (R&D Systems, Minneapolis, MN, USA)] and molecular marker were loaded onto NuPAGE® Novex 4% to 12% Bis-Tris (1.5 mm, 10 wells) gels (Invitrogen Life Technologies) and electrophoresed. The gels were later transferred onto polyvinylidine difluoride (PVDF) membranes by electrophoresis.

Twenty milliliters diluted anti-MMPs 1, 2, 3, 7, and 9 antibodies (R&D Systems) controls in a similar fashion with agitation. X-ray films were used to capture images which were later analyzed. Mean density values the protein bands were performed by densitometry on a Kodak Image Station 2000mm using Kodak Digital Science™ one-dimensional image analysis software (Rochester, NY, USA).

**Zymography on conditioned media**

Conditioned media from cultured HMCs treated with light chains were harvested at 12 hours, 48 hours, and 96 hours. These were diluted to contain 15 μg total proteins in 20 μL. Samples, recombinant human MMP-2 (2.5 ng/μL) and recombinant human MMP-9 (5.0 ng/μL) (R&D Systems) controls were further diluted in a similar volume of 2× Novex® Tris-glycine sodium dodecyl sulfate (SDS) sample buffer (Invitrogen Life Technologies). Twenty microliters of samples and controls were loaded onto Novex® zymogram (1.5 mm, 15 wells) gels (Invitrogen Life Technologies) and electrophoresed at 125 V constant for 90 minutes in Novex® Tris-glycine SDS running buffer (Invitrogen Life Technologies). After electrophoresis, the gels were removed and incubated in two changes of zymogram renaturing buffer (Invitrogen Life Technologies).
 Technologies) for 30 minutes at room temperature with gentle agitation. Gels were then washed in zymogram developing buffer (Invitrogen Life Technologies) then incubated in fresh developing buffer at 37°C overnight with gentle agitation. The gels were rinsed in distilled water then placed in Bio-Safe™ coomassie (Bio-Rad Laboratories, Hercules, CA, USA) for 40 minutes at room temperature and gentle agitation. They were then washed in three changes of distilled water for 20 minutes each. Gels were photographed and analyzed using a Kodak Image Station 2000mm and Kodak Digital Science™ one-dimensional image analysis software.

**Immunohistochemical analysis of MMPs, collagen IV, and tenasin**

Sixty-one formalin-fixed and paraffin-embedded kidney tissues from autopsy and renal biopsies from patients with different diagnoses (LCDD = 12, AL-Am = 10, negative controls = 31, and TMA = 8), were sectioned at 5 μm thick. Additionally, 46 cases were selected from the cases mentioned (LCDD = 3, AL-Am = 9, negative controls = 29, and TMA = 5) for collagen IV and tenasin investigations. Sections were deparaffinized, hydrated, and antigen retrieval performed on them using moist heat by placing them in preheated 0.01 mol/L sodium citrate (pH 6.0) and further heating at 100°C for 25 minutes [33].

Endogenous peroxidase activity in the tissue sections and HMCs from the in vitro experiments was blocked by incubating them with DAKO® Peroxidase Blocking Reagent (Dako Cytomation, Carpinteria, CA, USA) containing H2O2 for 5 minutes. Slides were then rinsed three times with PBS (pH 7.4) and blocked with DAKO® Protein Block Serum-Free (Dako Cytomation) containing 0.25% casein in PBS containing carrier protein and NaN3 for 5 minutes at room temperature. TTBS was used to rinse slides three times prior to the addition of the primary monoclonal antibodies. A total of 150 μL of diluted anti-MMPs 1, 2, 3, 7, and 9 (1:20 dilutions of 200 μg/mL stock) (R&D Systems) and collagen IV, 1:40 dilution (2.5 μg/mL) and tenasin-C, 1:40 dilution (14 μg/mL), were added to the slides. The slides were incubated at 4°C overnight, and then rinsed three times with TTBS. A total of 150 μL of peroxidase-labeled polymer (Dako Cytomation) was added to each slide and incubated for 30 minutes at room temperature. Sections were carefully rinsed three times with TTBS and drained. Slides were treated with chromagen substrate containing diaminobenzidine (DAB) then incubated at room temperature for 7 minutes. Slides were rinsed three times in distilled water followed by counterstaining with Harris’ hematoxylin for 10 seconds at room temperature. They were then placed in running tap water for 30 minutes. Sections were finally dehydrated through ascending concentrations of ethanol, cleared in xylene, then mounted with permount.

**Grading of immunohistochemical staining**

Immunohistochemical staining was assessed based on the presence or absence of brown coloration in the cells denoted as positive or negative, respectively. Staining was graded on a numerical scale of 0 to 4, where 0 represented no staining (negative), and 1 to 4 represented positive staining (1 being the least intense and 4 being the most intense staining). Brown staining intensity was assessed by comparison with the staining intensity of the nuclei present in the samples, and grading reflected the strongest staining intensity noted in the sample. Grading was conducted by two investigators independently and without knowledge of sample category.

**Protein and RNA isolation following laser capture microdissection (LCM)**

LCM technique was used to obtain intact glomeruli from formalin-fixed paraffin-embedded tissues. Two samples were selected from each of five specimen categories: ATN, AL-Am, negative controls, LCDD, and TMA. Two caps were used to harvest approximately 120 glomeruli each. One was used for protein and the other for RNA analyses. The caps earmarked for protein determination were inserted onto microcentrifuge tubes containing 50 μL T-PER™ tissue protein extraction reagent, inverted, and incubated for 30 minutes at room temperature. Samples were centrifuged at 10,000 rpm for 5 minutes and the supernatants collected. These were analyzed for MMPs 1, 2, 3, 7, and 9 proteins using Quantikine® ELISA assays (R&D Systems).

**Quantikine® ELISA assay for MMP proteins**

Supernatants obtained from LCM glomeruli were analyzed for MMP protein expressions using an ELISA technique. For normalization purposes, each sample was diluted to contain 1 μg of total protein. Kits were obtained for MMPs 1, 2, 3, 7, and 9 (R&D Systems). All reagents, working standards and samples were prepared in accordance with the manufacturer’s protocol. A total of 100 μL of assay diluent was added to each well of the supplied 96-well microtiter plate. Fifty microliters or 100 μL standard or sample was added to each well (depending on the requirements of the protocol for each MMP) and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker at 500 rpm. The contents of each well were aspirated then the wells were washed four times with wash buffer. Then, 200 μL conjugate was added to each well prior to incubating for 2 hours at room temperature on the shaker. The contents of each well was aspirated and washed four times with wash buffer then 200 μL substrate solution was added to each well. Plates were incubated for 20 or 30 minutes (depending on protocol) at room temperature in the dark. Finally, 50 μL
stop solution was added to each well and the absorbance at 450 nm and 540 nm for λ correction obtained.

RNA analysis using microarray technology

Total RNA isolated from treated HMCs (in vitro experiments) and laser-captured microdissected glomeruli were assessed for MMP and TIMP mRNA. Sample volumes were adjusted to contain a total RNA concentration of 1 μg (cultured HMCs) and 3 μg (glomeruli) in 18 μL with RNase-free H2O. A selective MMP/TIMP Non-Rad Ampo-Labeling GEArray Original Series (SuperArray, Fredrick, MD, USA) microarray technique was employed in measuring mRNA concentrations in these samples adhering to manufacturer’s instructions. The results of the experiments were obtained from membranes provided with the kits which were scanned then converted and saved as gray scale TIFF files. These were later analyzed using a ScanAlyze (Dr. Michael Eisen, Stanford University, California) software to identify and determine mRNA expressions of MMPs and TIMPs.

Statistical analysis

Statistical analysis of the results, where stated, was conducted using the Wilcoxon sum ranked test for nonparametric two-way analysis of variance (ANOVA) at a confidence interval of $P < 0.05$.

RESULTS

MMP expressions in cultured HMCs by Western blot

Conditioned media from HMCs treated with light chains were harvested at 72 and 96 hours and probed for MMPs 1, 2, 3, 7, and 9 using Western blots. Bands shown in Figure 2A were observed to be more intense the supernatant of the AL-Am-light chain–treated cells compared to the LCDD-light chain cells. Densitometry confirmed these findings and revealed higher MMP levels present in the conditioned media from AL-Am-light chain–treated HMCs than in those treated with LCDD-light chains. There were statistical significantly higher protein expressions of MMP-1 ($\times 3.4$) and MMP-7 ($\times 3.6$) at 72 hours and MMP-1 ($\times 3.7$), MMP-7 ($\times 1.6$) and MMP-9 ($\times 5.3$) at 96 hours by HMCs treated with AL-Am-light chains compared those treated with LCDD-light chains (Fig. 2B).

Zymography on conditioned media

Zymograms were performed on conditioned media from HMCs treated with light chains were harvested at 12 hours, 48 hours, and 96 hours. Bands migrating at 60 kD in the test samples and the recombinant human MMP-2 (rhMMP-2) controls were noted. These digestion bands were considered to be MMP-2. Data analysis revealed that MMP-2 levels were significantly higher in the media from AL-Am-light–treated cells compared to LCDD-light chain–treated cells and control (Fig. 3). Only the AL-Am-light–treated supernatant resulted in values which were greater than control.

Immunohistochemical analysis of HMCs post-light chain treatment

MMP protein expression was analyzed in the HMCs incubated with light chains (Fig. 4). Cells harvested at 96 hours of incubation showed intracellular expression of only MMP-7. Control cells showed no intracellular MMP expressions.

Immunohistochemical analysis of MMP expression in glomeruli and all renal compartments

MMP protein expressions in all renal compartments were quantitated; however, glomerular expressions were the main focus. These proteins were expressed differently in the glomeruli (Fig. 5A) with statistically higher expression levels (6.4 times) in AL-Am glomeruli compared to those in LCDD cases (Fig. 5B). Significant differences in the average total MMP expressed in all renal compartments by the AL-Am and LCDD cases (Fig. 5B). No statistical significant differences were observed between the negative (PPE, normal, and ATN) and LCDD cases. Similarly, no difference was noted between AL-Am and TMA (positive control) cases. The results presented in Figure 5 are the averages of MMPs 1, 2, 3, 7, and 9 in both the glomeruli and all renal compartments (glomeruli, tubular interstitium, and vasculature) for each disease category.

Collagen IV and tenascin expression in glomeruli

Collagen IV and tenascin protein expressions in glomeruli were analyzed (Fig. 6A). Significant differences were noted in tenascin protein expressions among the AL-Am and LCDD cases (Fig. 6B). LCDD cases expressed three times more tenascin and two times more collagen IV than the AL-Am cases. However, both AL-Am and LCDD glomeruli showed decreased levels of collagen IV compared to the negative controls.

MMP protein expressions in LCM glomeruli using ELISA

MMP proteins in LCM glomeruli were analyzed using an ELISA technique. Data showed increased MMP expressions in AL-Am glomeruli compared to LCDD cases. Statistically significant differences in MMPs 1, 2, and 7 were noted between the two groups compared. Significant differences were also noted in MMPs 1, 2, and 3 between AL-Am and negative cases. MMP-2 was the most highly expressed of the MMPs in all test categories (Fig. 7). The patterns of expression already identified in
MMP and TIMP mRNA expressions in LCM glomeruli using ELISA

The glomeruli obtained from the LCM samples were analyzed for their MMP mRNA expressions. Statistically significant differences (six times more) were noted between the AL-Am and LCDD cases for all the MMPs, and, notably MMP-7 which was markedly decreased in the LCDD glomeruli (Fig. 8). TIMP-1 mRNA expression was less than TIMP-2. Overall, data indicates that MMP and TIMP mRNAs were inversely proportional to each other especially in the case of AL-Am. Values shown are normalized to those obtained for the negative control as shown by the "0" line.

MMP and TIMP mRNA expressions in HMCs incubated with ICS using ELISA

Analysis of the in vitro HMCs reported data showed MMP-2, MMP-3, and MMP-7 to be more highly expressed statistically in AL-Am–treated cells than LCDD-treated cells (Fig. 9). Data from other time points (not shown) gave similar results. TIMP-2 mRNA was also noted to be significantly higher than TIMP-1. Test results were referenced against normal values (0 line).

CONCLUSION

MMPs are a group of ubiquitous enzymes with a diverse repertoire of proteolytic activities, including the activation of other MMPs through cleavage their prodromains. Expression of MMPs by mesangial cells can be altered by a variety of conditions. Glomerulopathic
light chains from patients with plasma cell dyscrasias are associated with two well-defined clinicopathologic entities: LCDD and AL-Am which alter mesangial homeostasis through divergent avenues. Mesangial matrix accumulation occurs in LCDD driven by activation of TGF-β [34], while ECM replacement is consistent with AL-Am. Interestingly, TGF-β is decreased in the latter [31]. The present translational study defines the role that MMPs play in these conditions and correlate tissue findings with in vitro data in an effort to elucidate the pathogenesis of these conditions.

Mesangial cells are crucial in the pathogenesis of both above-mentioned conditions associated with plasma cell dyscrasias. Mesangial cells are embedded in the mesangial matrix and control its turnover by maintaining equilibrium between synthesis and degradation of matrix components. They also generate the excess ECM rich in tenascin present in the mesangial nodules seen in LCDD [34]. Renal amyloidosis begins in the mesangium and mesangial cells play a fundamental role in the formation of amyloid fibrils and eventual replacement of the native mesangial matrix (Fig. 10). While the mechanisms involved in matrix replacement are not well understood, mesangial cell behavior is modulated by ECM components [13, 35, 36]. Relatively, phenotypic transformations occurring in these cells in relation to light chain type (AL-Am or LCDD) may also have a role in the overall response glomerular response to injury [37].

Due to the complexities and dynamism of HMCs and ECM behavior, coupled with the undefined roles of MMPs and TIMPs, this study was designed to investigate the behavior of HMCs in vitro. A comparison of these events with the glomerulopathic manifestations of AL-Am and LCDD could provide a basis for a translational approach to the understanding of their pathogeneeses.

The role of MMPs and TIMPs in renal diseases has been addressed by relatively few studies [10, 11, 38, 39]. Most of these studies have investigated MMPs 2 and 9 (gelatinases) which engage predominantly in the degradation of collagen IV, the most important native ECM protein in the mesangium. MMP-2 (gelatinase A) has been shown to regulate glomerular mesangial cell proliferation and differentiation [10] and play an important role in renal tubular epithelial-mesenchymal transformation [40]. The current availability of MMP-deficient mice has provided a new platform to further study the role of MMPs in glomerular homeostasis [41].

Two studies have addressed MMPs and TIMPs in amyloidosis [42, 43]. The first study analyzed the distribution of MMPs 1, 2, 3, and 9 and TIMPs in generalized amyloid A protein-associated amyloidosis (AA) and AL-Am in autopsy material [42]. Only MMPs 1, 2, and 3 were present in AA-amyloid deposits and only TIMP-1 and TIMP-2 (no MMPs) were noted in AL-amyloid deposits. The authors hypothesized that MMPs participate in amyloidogenesis either by processing the precursor protein serum amyloid-A (SAA), by degrading amyloid deposits, or by remodeling the interstitial matrix after amyloid deposition. The authors also concluded that since no MMPs were associated with AL-amyloid deposits, it was unlikely that the presence of MMPs was important for tissue remodeling in this type of amyloidosis. In the second study, amyloid degradation by MMPs 1, 2, and 3 was tested and the three MMPs were able to cleave SAA and amyloid fibril proteins [43]. The authors claimed this to be the first study to show that human SAA and amyloid fibril proteins are susceptible to proteolytic cleavage by MMPs.

Similar studies were not conducted with AL-amyloid.

MMPs 1, 2, 3, 7, and 9 were selected for analysis in the present study. These MMPs are representative of the entire spectrum of MMPs in terms of their composition and respective catalytic capabilities with the exception of membrane-associated MMPs (MT-MMPs) which were not studied [44]. Also, reliable commercial antibodies to these MMPs were available, which is not the case for all MMPs. Expression of the above mentioned MMPs and TIMPs was determined in glomeruli and total renal parenchyma in tissue samples. Total renal parenchymal MMPs and TIMPs determination was performed to gain
Fig. 4. Matrix metalloproteinase (MMP) expressions in human mesangial cells (HMCs) incubated with light chains. Cells were harvested at 96 hours’ incubation. Note only MMP-7 was identified in HMCs. Abbreviations are: MCN, myeloma cast nephropathy; LCDD, light chain disposition disease; AL-Am, AL-amyloidosis. Immunohistochemistry [diaminebenzidine (DAB) as chromagen] ×200.

Fig. 5. Matrix metalloproteinase (MMP) expression patterns in renal glomeruli. (A) Different categories of light chain–mediated glomerular injury. Note significant increase in expression of all MMPs in AL-amyloidosis (AL-Am) and in thrombotic microangiopathy (TMA) (positive control). LCDD is light chain disposition disease. Immunohistochemistry [diaminebenzidine (DAB) as chromagen] ×400. (B) Average glomerular and total (all renal compartments) expressions of MMPs in renal tissue by manual grading. Note the statistical significant differences between the AL-Am, LCDD, and the negative controls in glomerular (*) and all renal compartments (#).

A better understanding of the comprehensive activities of these molecules on renal homeostasis, in spite of the fact that this study was focused on glomerular alterations. Indeed, overall MMP expressions were markedly increased in renal tissues from patients with AL-Am when compared with negative controls and cases with LCDD.

The study primarily encompassed glomerular determination of MMPs and TIMPs expression using immunohistochemistry, protein quantification by ELISA techniques, and mRNA levels using a selective microarray for MMPs and TIMPs. LCM was employed to selectively obtain glomeruli from 12 patient samples, including
renal biopsies and autopsy specimens, from patients with LCDD and AL-Am. MMP and TIMP protein and mRNA levels were also assessed and in human mesangial cells incubated with light chains purified from the urine of patients with LCDD and AL-Am. A marked increase in MMP glomerular expressions in kidney specimens from patients with AL-Am compared with normal kidneys was noted with MMPs 1 and 7, revealing an eightfold increase and a 2.3-fold increase for MMP-2. There were no detectable intracellular expressions of MMPs in HMCs incubated with glomerulopathic light chains by immunohistochemistry, except for MMP-7. The cytoplasmic expression of MMP-7 was considered significant as the concentrated intracellular localization of any MMP was not expected. Previous attempts to detect MMP proteins in cell lysates were unsuccessful, while the proteins were abundantly found in the conditioned media, indicating that MMPs were secreted rapidly into the media. The presence of MMP-7 in intracellular vesicles suggests a defect in the release of this particular MMP into the ECM. This fact may be of significance in the pathogenesis of these disorders and may explain the tenascin-rich matrix that is typically noted in LCDD.

There were no significant differences in glomerular TIMP expressions in the two diseases when compared with control samples. There was, however, a significant decrease in TIMP-2 mRNA when compared with TIMP-1.
mRNA in microdissected glomeruli. Zymography was then used to confirm increased MMP-2 activity in AL-Am when compared with LCDD and normal. This finding indicates higher levels of active MMP-2 in the conditioned media of AL-Am-light chain–treated HMCs compared to LCDD-light chain cells.

Another striking finding was a marked reduction in MMP-7 in HMCs incubated with LCDD-light chain. Tenascin is known to be catabolized predominantly by MMP-7 and to a much lesser extent by MMP-3 and MMP-1 [47]. Tenascin is an ECM protein that is a component of the mesangium in normal and, most important,
pathologic conditions [48, 49]. It has been shown to accumulate in a variety of conditions resulting in irreversible functional consequences [50–52]. It has been demonstrated that there are a number of spliced tenasin variants [53–55], some very resistant to catabolism. Tenasin is known to accumulate in the center of mesangial nodules in patients with LCDD and nodular glomerulosclerosis and is also found in the mesangial matrix that remains in AL-Am [35]. Tenasin production by mesangial cells is enhanced in the in vitro model [18] as well as in patients with LCDD. The increased tenasin production and inhibited expressions of MMPs 7 and 3 combine to provide a clear understanding of the mechanisms at play in the alterations of mesangial matrix that occur in LCDD.

The results in this study indicate good correlation between in vitro and in vivo models for MMP and TIMP expression. It was demonstrated that human mesangial cells incubated with light chains from patients with AL-Am up-regulate MMP mRNA synthesis without concomittal up-regulation of TIMP mRNA synthesis. However, in LCDD-light chain–treated cells, MMP mRNA was down-regulated with no significant changes in TIMP mRNA synthesis. These in vitro results paralleled those from the microdissected glomeruli. The results observed in the in vitro system provided experimental support for MMP and TIMP alterations that have been documented to occur in LCDD and AL-Am [56]. Also, these findings provide evidence of the pathogenesis of these two glomerulopathies. The overproduction of MMPs with no increased inhibition of TIMPs results in the accelerated destruction of the mesangial matrix seen in AL-Am, while the inhibition of MMPs with normal
TIMPs production allows for the increased accumulation of matrix proteins seen in LCDD.

Evidence for the latter is further borne out in the data obtained from the analysis of collagen IV and tenascin in these glomeruli. The expressions of both proteins were noted to be increased in LCDD glomeruli with significant increased expression of tenascin compared to AL-Am glomeruli. The data obtained in the studies where HMCs were incubated on different matrices (data not shown) [abstracts; Keeling J and Herrera GA, Mod Pathol 18:267A, 2005; Lab Invest 85:267A, 2005] demonstrated further enhancement of the deleterious effects of the LCDD-light chains with incremental mesangial matrix protein production especially collagen I and tenascin. Collagen I is not present in the normal mesangial matrix. In LCDD the initial inhibition of MMP-7 promote the accumulation of tenascin in the mesangial matrix, potentiating further matrix increase. In contrast, overexpression of virtually all MMP proteins occurred when HMCs were incubated in the various matrices.

There are particular physicochemical characteristics of some light chains that make them prone to be associated with LCDD or AL-Am [24–29]. These peculiarities in the amino acid composition and the conformation of such light chains have been shown to play a key role in determining how they will affect function of mesangial cells and eventually alter mesangial homeostasis [21–29]. There may also be other factors, including host factors, which may also contribute to the propensity of a given light chain to affect mesangial cell function in a specific manner [57].

In summary, the present study depicts crucial pathogenic mechanisms involved in mesangial alterations that occur in LCDD and AL-Am. The study provides a platform for additional studies dealing with possible therapeutic interventions that would help stop, ameliorate or reverse adverse irreversible consequences arising from glomerulopathic mesangial cell interactions responsible for the pathologic consequences that occur in these disorders.

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REFERENCES


43. Muller D, Roessner A, Rocken C: Distribution pattern of matrix metalloproteinases 1, 2, 3, and 9, tissue inhibitors of matrix metalloproteinases 1 and 2, and alpha 2-macroglobulin in cases of generalized AA- and AL amyloidosis. Virchows Arch 437:521–527, 2000


