Changes in Matrix Metalloproteinases and Their Inhibitors in Kidney Transplant Recipients

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Abstract

Objectives: **Chronic allograft nephropathy remains one of the main causes of late graft loss after kidney transplant owing to multifactorial development of kidney scarring. Chronic allograft nephropathy is characterised by an excess accumulation of extracellular matrix. A key system regulating extracellular matrix homeostasis are matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases. This study sought to determine if a change in the matrix metalloproteinases/tissue inhibitors of matrix metalloproteinases system contributes to chronic allograft nephropathy-associated progressive kidney scarring.**

Materials and Methods: **Examination of sequential renal biopsies was done at implantation, acute rejection, and subsequent chronic allograft nephropathy. In situ localisation of matrix metalloproteinase activity was assessed with a high-resolution in situ zymography technique using gelatin and collagen 1 substrates. Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases were localised using immunohistochemistry.**

Results: **In situ zymography showed over a 50% reduction in matrix metalloproteinase activity against both collagen 1 and gelatin substrates in chronic allograft nephropathy biopsies. A similar loss of matrix metalloproteinase activity was seen in the glomerular and tubulointerstitial compartments. Immunoreactive matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases were observed intracellularly in mesangial and tubular epithelial cells. Matrix metalloproteinases-1, -2, -3, and -9 were significantly reduced in acute rejection and later in chronic allograft nephropathy. However, glomerular matrix metalloproteinases 1 and 9 and tubulointerstitial matrix metalloproteinase-2 were reduced at implantation. Tissue inhibitors of matrix metalloproteinase-2 and -3 were elevated from implantation onwards. We were unable to stain reproducibly for tissue inhibitors of matrix metalloproteinase-1.**

Conclusions: **Kidney scarring underlying chronic allograft nephropathy is associated with a reduction in matrix metalloproteinases activity that may be due to reduced expression of matrix metalloproteinases -1, -2, -3, and -9, and up-regulation of tissue inhibitors of matrix metalloproteinases -2 and -3. Some of these changes originate from implantation.**

Key words: *Matrix metalloproteinases, Tissue inhibitors of matrix metalloproteinases, Kidney scarring, Kidney transplant.*

Introduction

Kidney transplant is currently the most effective replacement therapy for patients with end-stage renal disease. Survival after renal transplant is better compared with age-matched individuals remaining on the transplant waiting list.[1, 2] Kidney transplant has a good outcome, giving a better quality of life,

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especially to young persons.[3, 4] During the last decade, renal allograft survival rates have increased significantly at 1 year after transplant.[5, 6] This is related to improvements in tissue typing, better understanding of immunology of the transplant, the use of more-potent immunosuppressive regimens, and better clinical management of recipients preoperatively and postoperatively.[7, 8] Currently, many centres are achieving acute rejection (AR) rates below 10%.[9] A major limitation in kidney transplant is the shortage of kidneys, particularly from deceased donors, which creates long transplant waiting lists worldwide.[10] Prolonging allograft survival by slowing the progression of the failing transplanted kidney is an area of significant interest.

Chronic renal allograft nephropathy (CAN) is the most-common cause of chronic graft dysfunction leading to graft failure.[11] There remains no effective therapy to slow or reverse the histologic damage associated with CAN.[12] The precise mechanisms responsible for the histopathologic changes seen in CAN, and whether progression of CAN to end-stage renal disease is different from progression of native chronic kidney disease, remain unclear. It is generally accepted that both alloantigen-dependent and -independent factors influence development of CAN.[13] The pathogenesis of CAN starts with an early insult; this can be through immunologic or nonimmunologic mechanisms and is followed by vascular remodeling owing to repetitive cycles of cytokine release, up-regulation of growth factors, and vascular smooth muscle cell proliferation that leads to typical concentric arteriosclerosis and ischemia, ending by an excessive accumulation of extracellular matrix (ECM) proteins in the glomeruli and tubulo-interstitium. Increased levels predominantly of collagen types 1, 3, and 4 lead to glomerulosclerosis, tubulo-interstitial fibrosis, and graft failure.[14]

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play a central role in maintaining the ECM homeostasis and thus, ECM remodeling and accumulation. Matrix metalloproteinases are a family of more than 24 members, with MMPs 1, 2, 3, 8, 9, and 13 having significant renal expression.[15] Matrix metallo - proteinases 1, 8, and 13 are interstitial collagenases degrading collagens 1 and 3; whereas MMP-2 and -9 are gelatinases that have collagen 4, fibronectin, and laminin amongst their preferred substrates. Matrix metalloproteinases-3 is a stromelysin that favours basement membrane ECM proteins, particularly fibronectin.[16] The kidney also expresses 3 tissue inhibitors of MMPs: TIMP-1, -2 and -3.[17] Tissue inhibitors of metalloproteinase-1 and 22 inhibit by blocking the MMPs' catalytic core, while TIMP-3 binds to the ECM and protects from MMP attack.[16] It is surprising that there is a paucity of data on the role of these enzymes and their contribution to the pathogenesis of CAN in comparison to the bulk of studies delineating their role in chronic kidney disease.[18] Limited studies have measured serum proMMP-1, proMMP-2, and proMMP-3 in acute renal allograft rejection, and CAN as a reflection of stimulation induced by several inflammatory cytokines in progression of CAN.[19, 20] None of the studies have located MMP/TIMP members in CAN, or, more importantly, assessed the net proteolytic activity in different compartments (glomerular vs tubulo-interstitial) of the transplanted kidney. We have recently localised MMP activity in experimental chronic kidney disease using a high-resolution, high-sensitivity in situ zymography technique. Differential activity between the tubules and the glomeruli was seen. Tubulo-interstitial remodeling was associated with a low ECM proteolytic state, whereas glomerulosclerosis was linked to large increases in MMP activity.[21]

We investigated the role of the MMP/TIMP system in the development of CAN by comparing its expression at implantation, in early AR episodes and subsequently, when CAN develops. We have applied this in situ zymography technique using whole molecule substrates to localise net MMP activity on tissue sections and complemented this with immunohistochemical localisation of the main renal MMPs and TIMPs to determine those responsible for changes in proteolytic activity in each compartment.

Materials and Methods

We undertook a retrospective analysis of adult kidney transplant recipients of deceased donors at the Sheffield Kidney Institute, Northern General Hospital, Sheffield, United Kingdom, from January 1, 2000 to December 31, 2002. Transplanted patients who underwent a renal allograft biopsy during their early posttransplant period (3 months) and had histologic diagnosis (biopsy proven) AR according to Banff 97 classification were identified. From those patients, we included only those who later on had a subsequent

renal allograft biopsy after the first 3 months that showed the histologic diagnosis of CAN, or dysfunction or chronic rejection according to Banff 97 classification. Only 16 patients (mean age, 40 ± 2.7 y) had adequate biopsy material sufficient for the inclusion criteria. Eight patients were men and 8 were women (mean age of deceased donors, 47 ± 2.9 y; 10 donors were men and 6 were women).

An implantation biopsy (donor or "zero-hour biopsy") was obtained from the deceased-donor kidney immediately after vascular anastomosis to assess the histologic and biochemical baseline of the donor kidney. These were taken as a wedge biopsy and formed part of the standard clinical practice at Sheffield Kidney Institute. Early biopsies (within the first 3 months) were obtained whenever there was an unexplained elevation of serum creatinine by at least 30% above the baseline. Patients were immunosuppressed by a triple-therapy protocol including 500 mg methylprednisolone in the operating theatre, followed by cyclosporine (6 mg/kg body weight in 2 divided dosages adjusted by whole blood level measurements using high-pressure liquid chromatography), azathioprine 1 to 2 mg/kg/d adjusted by whole white blood cell count, and prednisolone 20 mg/d. Acute rejection episodes were treated with intravenous methylprednisolone, or, if steroid resistant, with anti-thymocyte globulin. Proteinuria was measured and quantified by 24-hour urine collection for those who had positive urine dipstick for protein at the time of AR. The patients' demographic (age and sex), clinical, and biochemical data were collected from the Sheffield Kidney Institute database.

Kidney control tissue was taken from 5 patients who underwent a uninephrectomy for renal cell carcinoma and whose healthy renal pole was considered "normal" by the histopathology department at the Northern General Hospital, Sheffield. All protocols were approved by the ethics committee at the Sheffield Teaching Hospitals before the study began. Written, informed consent was obtained from all patients when possible. All protocols conformed with the ethical guidelines of the 1975 Helsinki Declaration.

Assessment of kidney scarring and deposited extracellular matrix proteins

Kidney scarring was determined on formal calcium fixed, paraffin-embedded sections (4 μm) that were stained using Masson's trichrome. Scarring was assessed by determining the percentage of green (collagenous) staining using a standard point counting approach.[22] Sections were examined at ×400 and ×200 magnifications for glomeruli and tubulo-interstitium using a 120-square grid (100 point) for the glomeruli and 25-point grid for tubulo-interstitium. Thirty glomeruli and 12 consecutive nonoverlapping cortical fields (area, 0.625 mm^2) were used for the analysis. Masson's trichrome was supported using immunohistochemical staining for α-smooth muscle actin (α-SMA) (myofibroblasts), collagen 3 and 4.

In situ zymography

To detect the location of the ECM proteolytic activity within normal and allograft kidney sections, a modified in situ zymography approach was used on fresh cryostat sections using DQ labeled substrates as previously described.[21] DQ Gelatin (ED-12054) (Molecular Probes, Cambridge Bioscience Ltd, Cambridge, United Kingdom) was used as a substrate that could be degraded by all MMPs, while DQ Collagen I (D-12060) was used as an interstitial MMP substrate for MMP-1, -8, and -13. In DQ labeling, the molecule is randomly labeled with FITC and a quenching agent. Cleavage of the substrate molecule results in separation of the quencher from the FITC, resulting in an increase in fluorescence. To limit diffusion of the cleaved substrate and hence, increase resolution, we applied the substrates in a 7% polyacrylamide gel. To achieve this, 250 μL of a 1 mg/mL solution of either DQ gelatin or DQ collagen 1 (molecular probes) was placed into 1 mL of a 30% acrylamide solution (37.5 acrylamide:1 Bis acrylamide). To this was added 3.9 mL of activation buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, and 0.2 mM NaN_3). The acrylamide was then encouraged to polymerase by addition of 50 μL 10% ammonium persulfate and 4 μL tetramethylethylenediamine. Two hundred microliters of this solution was placed on top of a fresh cryostat section, overlaid with a coverslip, and placed at 37°C until the polyacrylamide gel set, at which point the edge of the cover slip was immersed in activation buffer. Slides were placed in a humid chamber at room temperature away from light, and activation buffer was replaced every 12 hours to prevent the section from drying. Sections were left for 72 hours, and then all were examined under a fluorescent microscope for changes in fluorescence

resulting from substrate cleavage. The synthetic MMP inhibitor 1-10 phenanthrolene (50 μM) was used to verify the contribution of MMPs by incorporating it into the gel and activation buffer. Fluorescence was measured using phase analysis by calculating the area of positive fluorescence above a preset threshold using the AnalySIS 3.2 image analysis system (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Immunohistochemistry

A solution of 10% neutral buffered formalin-fixed and paraffin embedded 4-μm sections were dewaxed, hydrated, and had endogenous peroxidase quenched using 3% hydrogen peroxide in methanol. Epitopes were unmasked specifically for each antibody using either Antigen Unmasking System (ID Labs, Inc., Glasgow, UK), 0.5% (w/v) trypsin,[23] or microwaving in citrate acid buffer (9.99 mM, at pH 6) for 10 minutes.[23] Sections were blocked with 3% normal serum from the species in which the secondary antibody was raised (diluted in PBS/0.1% bovine serum albumin) for 45 minutes at room temperature. The primary antibody (or nonimmune serum) was applied at an optimised dilution overnight at 4°C.[21] Binding was revealed using a biotinylated anti-species antibody (45 minutes at room temperature) with the VECASTAIN ABC peroxidase kit and the substrate 3′-amino-9-ethylcarbazole (Vector Laboratories Inc, Burlingame, CA, USA). Sections were counterstained in hematoxylin (1:10) and mounted in glycerine (Dakopatts, UK).

Statistical analyses

Comparison between 2 quantitative variables was done using the *t* test, using a paired test for dependent and unpaired test for independent quantitative parametric variables. Data are presented as means ± SEM. The correlation coefficient test (r-test) was applied to assess the significance of the correlation between 2 variables (Pearson for parametric variables). Linear regression and multivariate Cox regression were used to assess the effect of different parameters on graft survival. Values for $P < .05$ were considered statistically significant. The coefficient of variance for immunohistochemistry and in situ zymography were 4% and 5.5%. Statistical analyses were performed with SPSS software (SPSS: An IBM Company, version 14.0, IBM Corporation, Armonk, New York, USA) and Microsoft Excel for Windows 2007 software.

Results

General observations

Mean cold ischemia time was 20 ± 2 hours. The average mean arterial blood pressure was 106 ± 1.2 mm Hg, cyclosporine levels were 107 ± 8 mg/d at the time of the biopsy showing CAN. Mean serum creatinine at the AR episode was 201.8 ± 12.2 μmol/L and at biopsy CAN was 279.8 ± 25.7 μmol/L. Twenty-four–hour proteinuria at AR was 52 ± 14 mg/d and 100 ± 38 mg/d at the time of the biopsy showing CAN.

Interstitial fibrosis was detectable in biopsies diagnosed with AR and advanced in CAN biopsies compared with normal and implantation (Table 1). To support this, sections were further stained for α-SMA, deposited ECM proteins collagen 3 and 4. α-SMA was only detected within the media of the renal arteries and arterioles in normal and implanted kidneys sections. However, in AR and particularly in CAN, there was a significant increase in α-SMA immunostaining in the interstitium and periglomerular areas and compared with normal and implantation biopsies. Both collagens 3 and 4 also were raised significantly in the interstitium and periglomerular in the AR and further, in CAN biopsies compared with normal biopsies.

Correlation of kidney function with renal allograft scarring markers

Both proteinuria and serum creatinine levels showed a strong correlation with fibrosis markers in CAN. Serum creatinine correlated positively with 24-hour proteinuria, interstitial fibrosis, α-SMA immunostaining, immunostainable collagen 3, and collagen 4. Proteinuria also correlated with interstitial fibrosis, α-SMA, collagen 3, and collagen 4. Creatinine clearance correlated negatively with 24-hour proteinuria, interstitial fibrosis, α-SMA, collagen 3, and collagen 4 (Table 2), the correlation of the deteriorated kidney function with the fibrosis markers indicated the progression of scarring in the CAN.

In situ zymography

Using gelatin and collagen 1 substrates, in a "normal" kidney, there was degradation within the glomeruli and tubules, with the greatest cleavage of gelatin within the tubular epithelial cells (Figure 1 A and D). This could be abolished by addition of the MMP inhibitor 1-10 phenanthrolene (Figure 1 F and I).

Table 1. Markers of Kidney Fibrosis and Scarring

	Interstitial Fibrosis	α-SMA	Collagen 4	Collagen 3
Normal	2.3 ± 0.6	0.68 ± 0.35	4.1 ± 0.98	0.32 ± 0.1
Implantation	3.15 ± 2.2	3.64 ± 1.9	9.9 ± 2.9	0.33 ± 0.06
AR	7.29 ± 1*	13.77 ± 3.8*	22.22 ± 1.4*	14.11 ± 4.5*
CAN	42.59 ± 7.1‡	16.51 ± 2.5†	34.8 ± 5.3†	17.11 ± 4.5†

Point-counting was used to quantify interstitial fibrosis, α-SMA, collagen 3 and 4 in biopsies diagnosed with AR and CAN in comparison to normal and implantation biopsies. Data represent mean ± SEM of point count (%), ‡$P < .0001$, †$P < .001$, *$P < .05$.

Abbreviations: α-SMA, α-smooth muscle actin; AR, acute rejection; CAN, chronic allograft nephropathy; SEM, standard error of the mean

Table 2. Correlation of Kidney Function With Renal Allograft Scarring Markers

Kidney Function	Interstitial Fibrosis	Collagen 3	Collagen 4	α-SMA	24-H Proteinuria	Serum Creatinine
24-h proteinuria	r= 0.48‡	r= 0.67‡	r= 0.40‡	r= 0.69‡	-	r= 0.489***
Serum creatinine	r= 0.53‡	r= 0.71‡	r= 0.71‡	r= 0.73‡	NS	-
Creatinine clearance	r= -0.55‡	r= -0.68‡	r= -0.65‡	r= -0.68‡	r= -0.359†	NS

Correlation between kidney function and fibrosis markers (interstitial fibrosis, α SMA, collagen 3, and collagen 4). Spearman's correlation coefficient test (r-test) for nonparametric variables was used to assess the significance of the correlation between 2 variables. Values for $P < .05$ were considered significant. r = Correlation coefficient. ‡$P < .0001$, †$P < .001$, *$P < .05$.

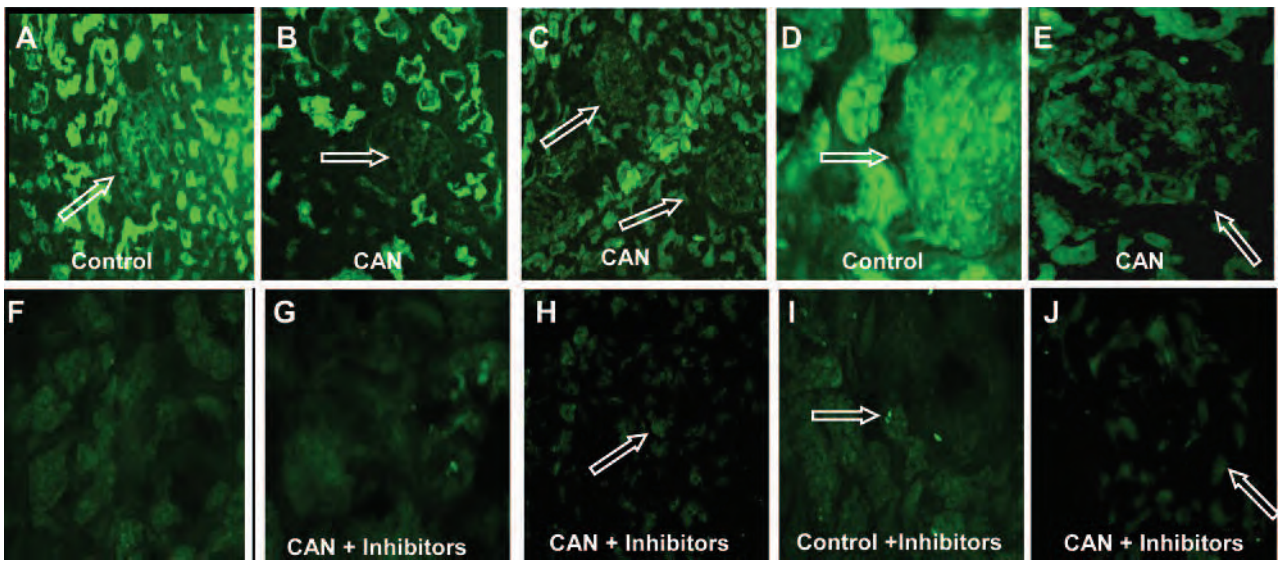
Abbreviations: α-SMA, α-smooth muscle actin; NS, nonsignificance

Precise areas of MMP activity within glomeruli are hard to identify owing to the requirement for cryostat tissue; however, proteolysis seems to occur in the glomerular basement membrane and mesangium. In CAN sections, there was a drop in activity in the tubular epithelium and the glomeruli compared with normal sections in both substrates (Figure 1B, C, and E). Measurable activity could be blocked by MMP inhibitor 1-10 phenanthrolene confirming that this was due to MMP activity (Figure 1G, H, and J). Of note, there was no significant breakdown of either gelatin or collagen 1 in the extracellular spaces and the interstitium, in either normal or CAN sections (Figure 1 A-E). The changes in proteolysis were quantified by multiphase image analysis. This showed a marked decrease in gelatin cleavage and thus, total MMP activity from normal levels in both the glomerular (-49%) and tubular compartments (-53%) in CAN (Figure 2B). Degradation of collagen 1 (interstitial collagenase activity) also showed similar reductions in the glomeruli (-51%) and tubulo-interstitium (-49%) (Figure 2A)

Immunohistochemical staining for the matrix metalloproteinases and their inhibitors

Matrix metalloproteinases and TIMP immunostaining in the glomerular and tubular compartments confirmed the in situ zymography location, with both being expressed predominantly intracellularly in the tubular epithelial cells of the proximal and distal tubules and collecting ducts. Glomerular expression also appeared intracellular in epithelial and mesangial cells. Hardly any extracellular (interstitial) staining was seen in any biopsies studied from normal, implantation, AR, and CAN (Figures 3 and 4).

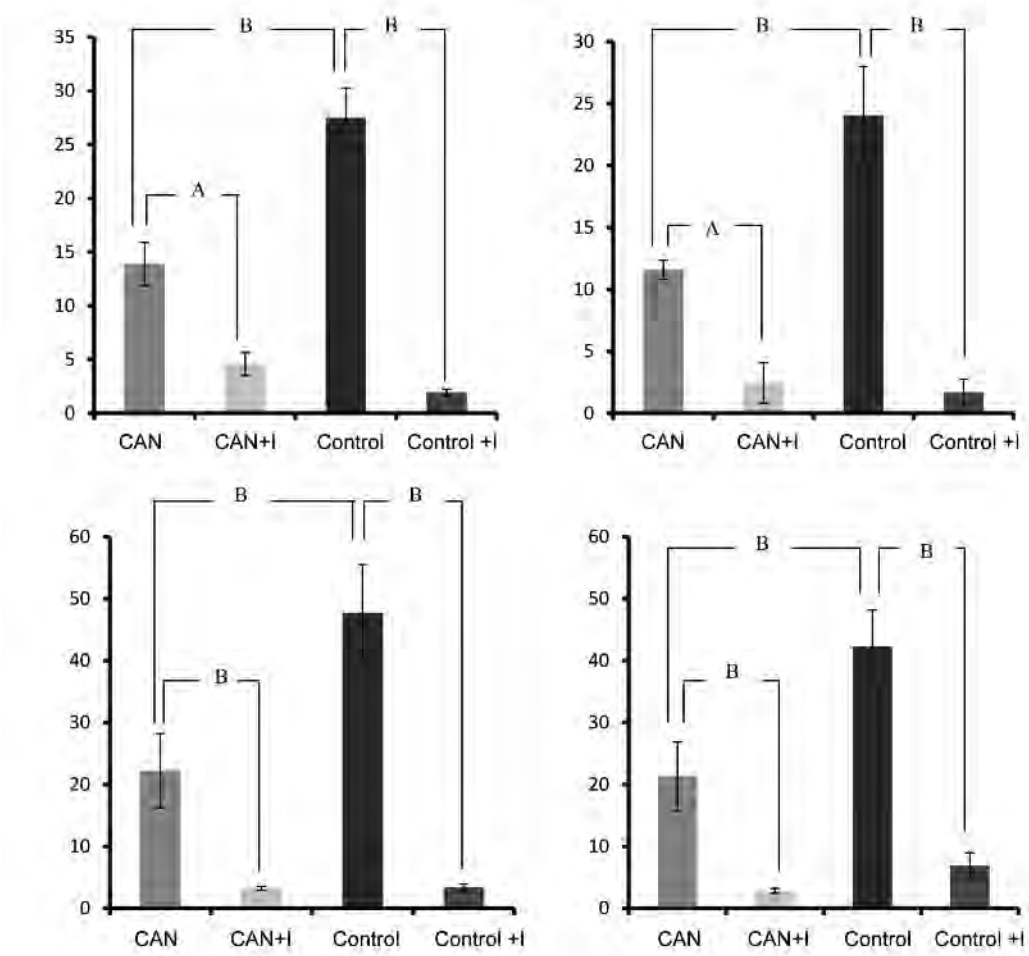
Figure 1. In Situ Zymography of Proteolytic Activity in CAN



Representative photomicrographs of in situ activity (bright green) using a DQ gelatin substrate in normal kidney (control) biopsies (A, D) and CAN (B, C, and E). Images of comparable normal and CAN (F-J) treated with the MMP inhibitor 1,10 phenanthrolene are shown on the lower row to demonstrate the level of activity resulting from MMP action. A, B, C, G, and H are ×200 magnification, D, E, I, and J are ×400 magnification. Arrows point at the glomerular compartments.

Abbreviations: CAN, chronic renal allograft nephropathy; MMP, matrix metalloproteinases

Figure 2. Quantification of In Situ Zymography Proteolytic Activity in CAN



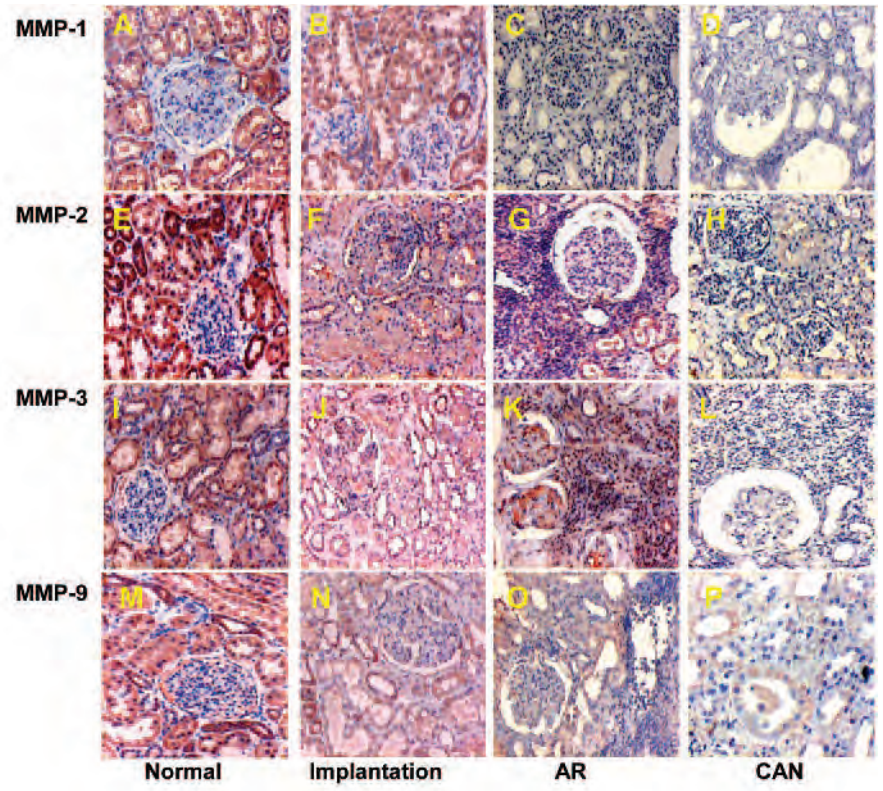
Analysis of MMP in situ zymography using both DQ collagen 1 (A) and gelatin (B) substrate in normal kidney and CAN in both the tubulo-interstitial and glomerular compartments. Percentage of fluorescence was measured using the AnalySIS 3.2 image analysis system. +I denotes addition of MMP inhibitor 1,10 phenanthrolene. At least 10 tubulo-interstitial fields or 10 glomeruli were analysed per section. Data represents mean percentage of the fluorescent area in $\mu m^2 \pm$ SEM (indicating proteolysis). A=$P < .05$, B=$P < .01$, C=$P < .001$.

Abbreviations: CAN, chronic renal allograft nephropathy; MMP, matrix metalloproteinases

Expression of all MMPs in both the tubular and glomerular compartments significantly fell in AR and CAN biopsies. Of note, was that glomerular MMP-1 was reduced by 72% compared to implantation. Subsequently, glomerular MMP-1 was barely detectable in AR (-93%) and CAN biopsies (-98%) with comparable tubular reductions in both AR and CAN (-93% and -88%) (Figures 3 and 5). Matrix metalloproteinase-9 showed similar large reductions in the glomeruli to MMP-1 with significant losses of MMP-9 evident at implantation and dropping by 82% reduction in CAN. However, in the tubulointerstitial compartment, there were no significant reductions in MMP-9. In contrast, the other gelatinase, MMP-2, showed much smaller reduction in staining in AR (-59%) and CAN (-51%) in the glomeruli, but was significantly lower in the tubulointerstitium from implantation onwards (implant -45%, AR -52%, CAN -58%). This demonstrates differential regulation between the individual gelatinases (Figures 3 and 5).

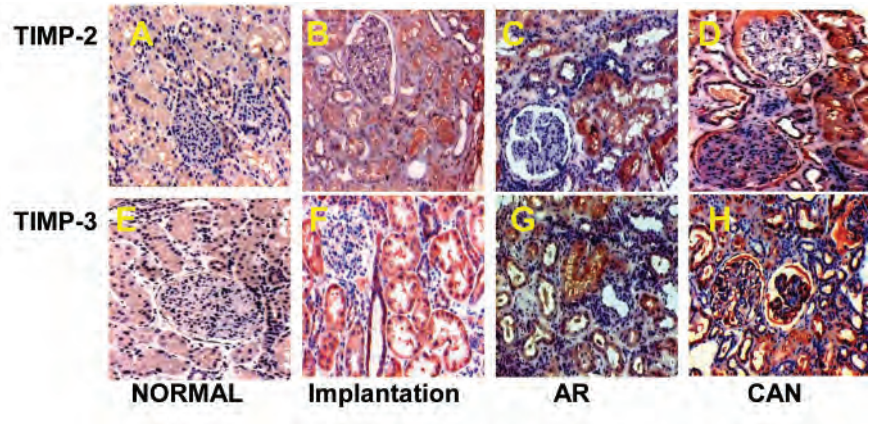
It was not possible to visualise TIMP-1 by immunofluorescence in any sections. Tissue inhibitors of metalloproteinase-2 and TIMP-3 were low in the normal kidney, especially in the glomeruli. However, both had increased staining in the implant biopsies, again more noticeably in the glomeruli with 91% and 90% for TIMP-2 and -3 (Figures 4 and 6). Both TIMPs seemed to be elevated mainly in mesangial cells and in proximal tubule cells. Both TIMP-2 and -3 levels remained significantly higher in AR and CAN than in normal kidney levels, but did not reach levels higher than at implantation (Figure 6).

Figure 3. Immunostaining of MMP in Acute Rejection and CAN



Representative photographs of MMP immunohistochemical staining in normal (A, E, I, and M), implantation (B, F, J, and N). Acute rejection (C, G, K, and O), CAN (D, H, L, and P) in both tubulo-interstitial and glomerular compartments ×200, and ×400 magnification.
Abbreviations: CAN, chronic renal allograft nephropathy; MMP, matrix metalloproteinases

Figure 4. Immunostaining of TIMP in Acute Rejection and CAN

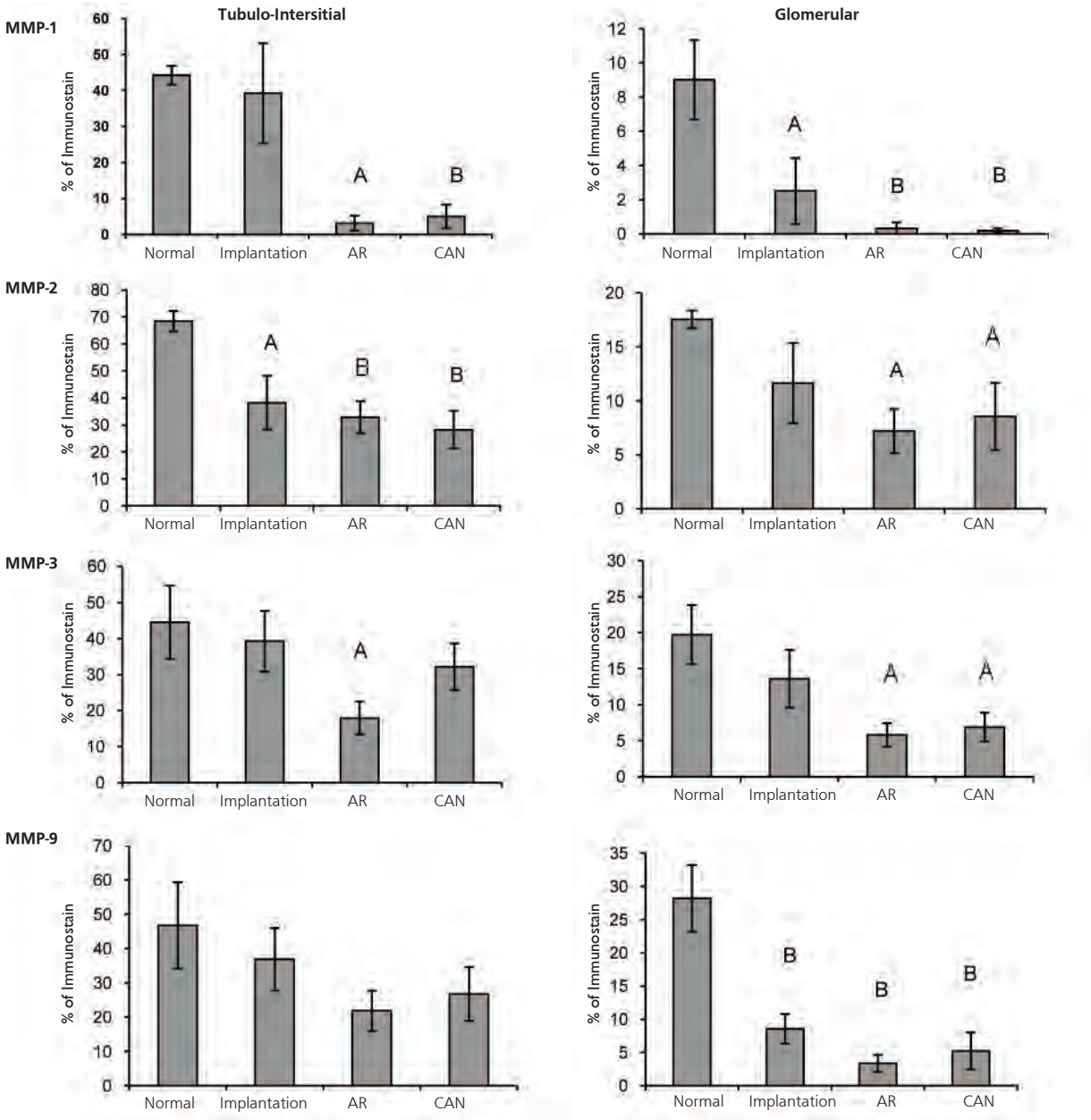


Representative photographs of TIMP immunohistochemical staining in normal (A and E), implantation (B and F), acute rejection AR (C and G), chronic allograft nephropathy CAN (D and H) in both tubulo-interstitial and glomerular compartments ×200, and ×400 magnification.
Abbreviations: CAN, chronic renal allograft nephropathy; MMP, matrix metalloproteinases; TIMP, tissue inhibitors of metalloproteinases

Predictors of kidney function and interstitial fibrosis

Linear regression analysis showed both collagen 3 expression and tubular expression of TIMP-3 at the time of AR, to be useful predictors of kidney scarring in CAN as indicated by the later interstitial fibrosis in the CAN biopsy ($P < .05$) (Table 3). Further, the mean 24-hour urinary protein excretion was a predictor of worsening kidney function as shown by 1/serum creatinine slope $P < .0001$ (Table 3).

Figure 5. MMP Immunostaining



Point-count analysis was used to quantify MMP staining in the tubulo-interstitium (×200 magnification) and glomeruli (×400 magnification). Twelve consecutive nonoverlapping tubulo-interstitial fields and a minimum of 10 glomeruli were analysed for each section. Data are shown as mean percentage change from normal. A=$P < .05$, B=$P < .01$, C=$P < .001$

Abbreviations: AR, acute rejection; CAN, chronic allograft nephropathy; MMP, matrix metalloproteinases

Correlation of collagenolytic activity and matrix metalloproteinases/tissue inhibitors of metalloproteinases

Tissue inhibitors of metalloproteinase-2 showed a strong negative correlation to in situ zymography activity (gelatin substrate) in CAN (r= -0.96; $P < .0001$). Tissue inhibitors of metalloproteinase-3 was also negatively correlated to MMP-1 levels in CAN (r= -0.68; $P < .01$) (Table 4).

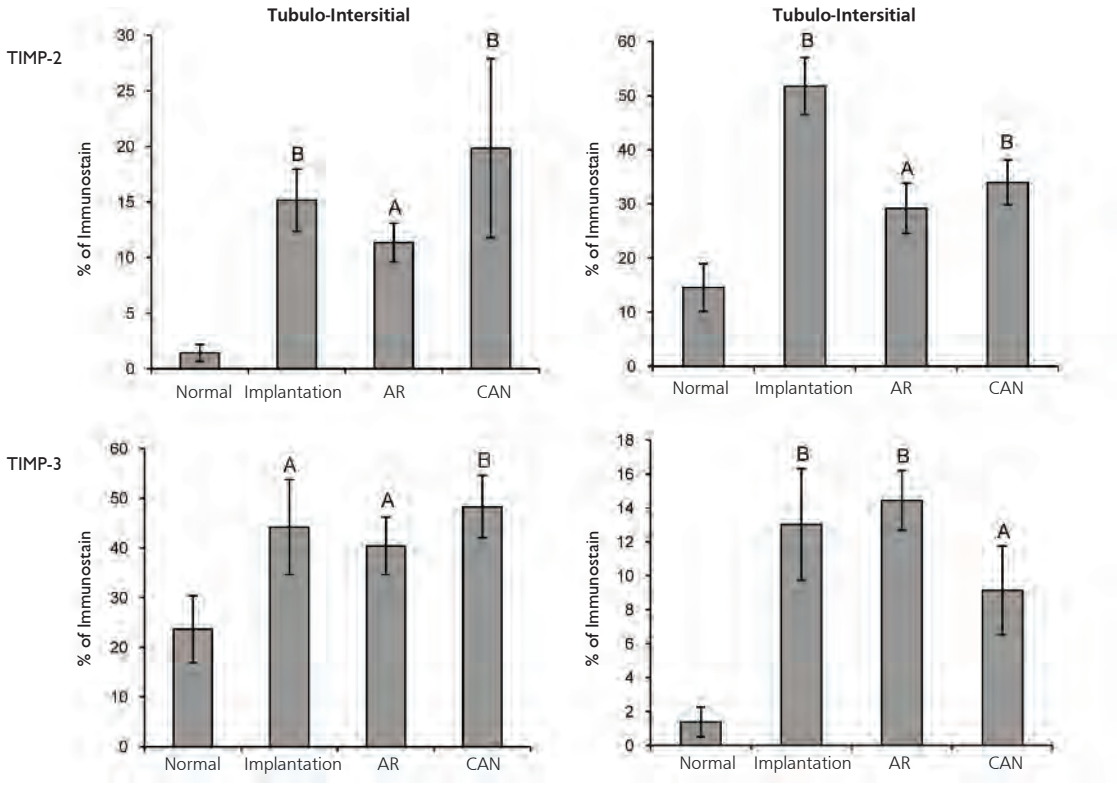
Table 3. Linear Regression Analysis for Different Variables Against Outcome (Kidney Function and Interstitial Fibrosis)

Parameter	Parameter	Significance	Beta	*t*
Average 24-h urinary proteins	1/Scr slope	‡	-1.27	
Collagen 3 staining	Interstitial fibrosis in CAN	*	0.41	2.25
TIMP-3	Interstitial fibrosis in CAN	*	0.76	2.325

Linear regression analysis showed both collagen 3 expression and upregulated TIMP-3, at the time of AR, to be useful predictors of kidney scarring in CAN as indicated by interstitial fibrosis at time of biopsy showing CAN. Average 24-hour urinary protein excretion was a predictor of worsening kidney function. ‡$P < .0001$, *$P < .05$.

Abbreviations: AR, acute rejection; CAN, chronic allograft nephropathy; Scr, serum creatinine; TIMP, tissue inhibitors of matrix metalloproteinases

Figure 6. TIMP Immunostaining



Point-count analysis was used to quantify TIMP staining in the tubule-interstitium (×200 magnification) and glomeruli (×400 magnification). Twelve consecutive nonoverlapping tubule-interstitial fields and a minimum of 10 glomeruli were analyzed for each section. Data are shown as mean percentage change of positive immunostaining from normal. A=$P < .05$, B=$P < .01$, C=$P < .001$

Abbreviations: AR, acute rejection; CAN, chronic alloograft nephropathy; TIMP, tissue inhibitors of metalloproteinases

Factors affecting the graft survival in chronic renal allograft nephropathy

Using Multivariate Cox regression to assess the effect of different parameters on graft survival, we found that mean systolic blood pressure, 24-hour proteinuria, and acute vascular and tubular score of Banff 97 had a significant negative effect on graft survival at the time of CAN biopsy. None of the MMP or TIMP measurements had an effect on graft survival at 5 years (Table 5).

Discussion

In this study, we have localised MMP activity for the first time in human kidney allografts using a sensitive high-resolution in situ zymography technique. We report a predominantly intracellular location of MMP activity and describe a progressive reduction in tubular and glomerular MMP activity with the development of CAN. To confirm that measured activity was due to MMP action, the broad-spectrum MMP inhibitor 1-10 phenanthrolene was applied, indicating that over 95% of substrate cleaved was due to MMP action. Parallel immunohistochemistry for individual MMPs and TIMPs confirmed a

Table 4. Correlation of Collagenic Activity and MMPs/TIMPs

Kidney Function	TIMP-2	TIMP-3	MMP-1	In Situ Zymography Activity to Gelatin
TIMP-2	-	NS	NS	r= -0.96‡
TIMP-3	NS	-	r= -0.68*	NS
MMP-1	NS	r= -0.68*	-	NS
In situ zymography activity to gelatin	r= -0.96‡	NS	NS	-

Correlation between kidney collagenic activity and MMPs/TIMPs. Spearman's correlations coefficient test (r-test) for the non-parametric variables was used to assess the significance of the correlation between two variables. Values for $P < .05$ were considered significant.

r = Correlation coefficient. ‡$P < .0001$, *$P < .05$.

Abbreviations: MMP, matrix metalloproteinases; NS, nonsignificance; TIMP, tissue inhibitors of matrix metalloproteinases

Table 5. Factors Affecting the Graft Survival (5-y) in CAN

Parameter	Regression Coefficients	*P* Value
Mean systolic blood pressure	1.34 (34%) 1.098-1.64	†
Average 24-h urinary protein	4.28 (328%) 1.04-17.48	*
Banff; acute vascular score	2.27 (127%) 1.15-4.48	*
Banff; acute tubular score	4.21 (321%) 0.97-18.43	*

The multivariate Cox regression prediction model was used to assess the effect of mean systolic blood pressure (MSB), 24-h proteinuria, and acute vascular and tubular score of Banff 97 on graft survival of deceased-donor CAN. †$P < .001$, *$P < .05$.

Abbreviations: CAN, chronic allograft nephropathy

predominantly intracellular location and also showed the reduced activity was due to reduced levels of most MMPs and up-regulation of both TIMPs measured.

Elevated TIMP-2 and -3 were observed from implantation onwards with levels remaining high in biopsies taken later for AR, and development of CAN suggesting that changes in TIMPs are triggered either in the donor or as part of the retrieval/implant process. In a similar manner, some MMPs, especially MMP-1 and MMP-9 in the glomeruli, were reduced in implant biopsies. Taken together, these data clearly show that development of CAN is associated with the same shutdown in MMP activity seen in native kidney fibrosis that leads to reduced ECM clearance, and that this shutdown is due to a combination of reductions in MMP levels, as well as increases in their natural inhibitors. Further, the data suggest that some changes are seen in the implant biopsies and thus, long-term perturbations in the ECM clearance may have their roots in the donated organ. However, no links were found between changes in MMPs or TIMPs at implantation with 5-year graft survival in this study, which suggests that these changes are not critical to graft failure.

We have previously located and visualised MMP activity in experimental kidney scarring where differential activity has been seen between the glomerular and the tubular compartments.[21] We reported that while tubulo-interstitial remodeling is associated with a low ECM proteolytic state, as here, glomerulosclerosis was linked to large increases in MMP activity owing to increased glomerular MMP-1, which would lead to remodeling of the glomerular basement membrane.[21] Clearly, this is not the case here, as both tubular glomerular compartments showed a shutdown in MMP activity as fibrosis developed in CAN. The reason for this difference is not immediately clear; however, it is almost certainly related to the different factors driving the fibrotic pathway, especially as the subtotal nephrectomy model used is a hypertensive model, whereas in CAN, there is a strong immunologic component. In saying this, an experimental model of CAN reports that early inhibition of MMP slows progression of CAN. However, if inhibition starts late, there is significant progression to CAN development. These results suggest that early events could have started from implantation or during early AR may lead to early rises in MMP activity, such as those associated with inflammatory cell invasion and penetration of basement membranes, thus damaging or altering composition of the ECM, while later loses in ECM turnover may lead to ECM accumulation.[18] Their data in an animal model does, however, conflict with what we report in this human study.

There is a considerable literature on TIMPs and MMPs in the scarring process, mainly from numerous animal models. Those that have measured MMP activity relate lower MMP activity to tissue scarring[24, 25] are in line with our data. However, lower MMP activity does not necessarily relate to lower production of MMPs, as we have previously demonstrated elevated levels of several MMPs at the mRNA level in the fifth and sixth SNx models of chronic kidney disease,[26] while in a later paper, demonstrated that this was not seen at the activity level.[21]

One of the most frustrating things in this study has been the inability to stain reproducibly for TIMP-1 owing to technical issues. In ELISA and Western blot studies, this is consistently the largest player in animal models of chronic kidney disease,[21, 26] and thus, highly likely to be regulated here. Hopefully, at some point, suitable antibodies will become available to stain for TIMP-1 in biopsy material and/or tissue can be made available for Western blot or mRNA analysis.

In contrast to animal studies, there are a limited number of reports of MMP/TIMP in human kidney disease, mostly they report an increase in TIMPs associated with ECM accumulation and progression of the underlying nephropathy, as we see here in CAN. Human kidney biopsies with diabetic nephropathy show elevated expression of TIMP-1 mRNA levels.[27] In addition, MMP-2 mRNA expression was found to be low in glomeruli of non-insulin–dependent diabetic patients, suggesting that reduced MMP production may play a pivotal role in matrix accumulation in diabetic nephropathy.[28] In situ hybridization on renal biopsy specimens from patients with IgA nephropathy showed raised MMP-3 and TIMP-1 mRNA expression in both glomerular cells and tubular epithelial cells.[29] It has been shown in human adult polycystic kidney disease that there is an increase in MMP-2 and -9 levels that was localised to tubular cells, with the greatest amounts in the collecting duct epithelia.[30] Further, expression of glomerular and interstitial MMP-2, -3, -9, and TIMP-1 was increased in active ANCA-associated glomerulonephritis and was shown to correlate with inflammatory activity.[31] Clearly, all these studies show

some inconsistencies included with the data we present here. This not only indicates the complexity of the MMP/TIMP system in vivo but that the MMP/TIMP system may be differentially responsive to the underlying disease making tackling perturbations in MMP activity in chronic kidney disease a difficult proposition. Comparative analysis of the multiple measurements in our study demonstrate that TIMP-2 negatively correlates with tubulointestinal MMP activity (gelatin substrate) in CAN, while TIMP-3 negatively correlates with MMP-1 levels demonstrating a coordinated response.

Elevated TIMP-3 also was a useful predictor of interstitial fibrosis in CAN biopsies. Other studies have reported consistent data. Nicholson and associates showed positive correlations between the level of tubulo-interstitial collagen 3 immunostaining and expression of genes for TIMP-1 and TIMP-2 in CAN.[32] Further, TIMP-1 expression increased with TGF-beta expression. Nicholson drew similar conclusions to those we report in that failure of ECM degradation, particularly due to elevated TIMPs, may be an important molecular mechanism in the pathogenesis of chronic renal allograft damage.[32]

In both our previous studies of MMP/TIMP in the subtotal nephrectomy model of kidney scarring, we also demonstrated that shutdown in MMP activity is driven by elevated TIMP levels and thus, overexpression of TIMPs is central to ECM accumulation.[21, 26]

However, not all data on MMPs and TIMPs is in line with this conclusion. Elevated serum levels of proMMP-1, proMMP-2, and proMMP-3 have been reported in patients with AR and CAN in 2 studies by Rodrigo and colleagues.[19, 20] High serum levels of the proMMPs could be a reflection of the stimulation induced by several inflammatory cytokines produced in the inflammatory response in AR. In addition, they also could play an important role in the pathogenesis of CAN[19, 20] if they became activated. Brook and associates observed that at the mRNA level, glomerular expression of MMP-2, TIMP-1, and TIMP-2 did not differ between renal graft recipients who experienced AR episodes and those who were free from rejection. However, they did not follow-up these patients to see whether or not they developed CAN later on, nor did they measure the mRNA levels of MMPs/TIMPs at implantation.[32]

Throughout the literature, attempts to visulalise MMPs and TIMP by immunohistochemistry both in the kidney and in other tissues, as well as our own attempts, including in situ MMP activity assays, have consistently demonstrated the vast bulk of MMPs and TIMPs in an intracellular cytoplasmic location.[22, 33-37] This conflicts with the perceived primary location ascribed to MMPs and TIMPs, which is based predominantly on in vitro cell culture studies that have positioned the MMP/TIMP system as extracellular owing to its function in ECM degradation and matrix remodeling as well as adhesion.[38, 39] Therefore, we would have expected to find both MMPs and TIMPs mainly in the interstitium. However, this study, as well as our previous work,[21] suggests a mainly intracellular location and subsequently, an intracellular role or mechanism for these proteins. This could be due to TIMPs and MMPs being stored intracellularly before export and a lack of sensitivity in visualization techniques once extracellular, but one cannot exclude an intracellular role.

In conclusion, for the first time in kidney scarring associated with CAN, we have been able to precisely localise changes in MMP activity that result from the balance of proMMPs, active MMPs, and TIMPs, without destroying the cellular compartmentalisation by homogenisation. Further, this has been combined with individual MMP and TIMP localisation to provide a clearer picture of in situ MMP action in CAN. The study clearly links low ECM proteolytic action with reduced MMP levels and elevated TIMP levels, which differ from several other studies in animal models and human studies including some of our own. Restoring MMP activity remains an attractive strategy for slowing tissue fibrosis, such as in CAN, although differences between studies makes this far from a clear interventional approach. The work presented here would clearly suggest that a TIMP lowering approach with elevation of some MMPs would likely be beneficial in slowing CAN.

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