

ORIGINAL ARTICLE

Renal dysfunction and intragraft proMMP9 activity in renal transplant recipients with interstitial fibrosis and tubular atrophy

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Conflicts of interest

None of the authors have any conflict of interest to disclose in relation to the study.

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Summary

Chronic renal allograft injury is reflected by interstitial fibrosis and tubular atrophy (IF/TA) and by the accumulation of extracellular matrix (ECM). Metalloproteinases (MMPs) are renal physiologic regulators of ECM degradation. Changes in MMPs expression or activity may disturb ECM turnover leading to glomerular scarring and worsening renal function. Our goal was to investigate intragraft MMP2 and MMP9 activities and their correlation with renal dysfunction. Plasma MMP2 and MMP9 activities were analyzed as noninvasive markers of renal allograft deterioration. Transplanted patients were biopsied and histopathologically characterized as IF/TA+ or IF/TA-. Renal function was evaluated by serum creatinine, glomerular filtration rate (GFR) estimated by Modification of Diet in Renal Disease equation and urinary protein/creatinine ratio. Kidney and plasma MMP2 and MMP9 activities were analyzed by zymography. A significant renal dysfunction was observed in IF/TA+ patients. Intragraft proMMP9 showed a significant higher activity in IF/TA+ than in IF/TA- samples and was inversely correlated with the GFR. Intragraft proMMP2 activity tended to increase in IF/TA+ samples, although no statistic significance was reached. Circulating proMMP2 and proMMP9 activities did not show significant differences between groups. Our data provide evidence that correlates intragraft proMMP9 activity with the fibrotic changes and renal dysfunction observed in IF/TA.

Introduction

During clinical renal transplantation, many renal allografts are lost in the long term due to chronic renal dysfunction associated with the development of graft fibrosis and glomerulosclerosis. This entity, previously called chronic allograft nephropathy (CAN), has been renamed as “interstitial fibrosis and tubular atrophy without evidence of any specific etiology (IF/TA)” [1]. The clinical diagnosis of IF/TA is suggested by gradual deterioration of graft function, manifested by slowly rising serum creatinine concentration, increasing proteinuria and worsening hypertension [2]. The pathologic changes of IF/TA involve all parts of the renal parenchyma including blood vessels, glomeruli, interstitium, and tubules [3], but the accumulation of extracellular matrix (ECM) is the histological hallmark of IF/TA and is responsible for progressive tubulo-interstitial fibrosis.

It has been generally accepted that the extent of fibrosis is a balance between net collagen synthesis and degradation, and that a reduction in protease activity can result in fibrosis. Nowadays, the presence of fibrosis due to a decrease in protease activity has been questioned [4,5]. In turn, renal fibrosis manifests as an accumulation of collagens in the kidney. Most collagen degradation in the kidney is controlled by matrix metalloproteinases (MMPs).

MMPs are endopeptidases that act on the ECM degradation. The expression of MMPs is regulated by a wide variety of compounds including inflammatory cytokines such as IL-1 [6] and growth factors such as TGF β [7]. A second level of control of MMPs activity is achieved through the secretion of tissue inhibitors of matrix metalloproteinases (TIMPs) [8].

Among MMPs, the gelatinases MMP2 and MMP9 have been extensively studied in kidney organogenesis [9,10] as well as in renal transplantation models for acute allograft rejection [11] and CAN [12]. In clinical studies, an increase in serum proMMP2 and proMMP3 levels associated with CAN has been described [13]. When intragraft markers were investigated, a positive correlation between tubulo-interstitial collagen deposition and intragraft expression of the genes for TIMP1 and TIMP2 was described [14], suggesting that alterations in the ratio between TIMPs and MMPs may be an important molecular mechanism leading to the development of tubulo-interstitial fibrosis. In relation to this finding, other authors described a positive correlation between gene expression of metzincins, zinc-dependent metalloproteases, and the severity of CAN [15]. However, the available information in human samples is limited and contradictory [16]. Scant studies focused in the intragraft expression of MMPs activity have been performed. In a CAN rat model, a decreased TIMP3 and increased proMMP2, proMMP9 and MMP2 levels in renal

allograft biopsies were described associated with increased interstitial collagen content [12]. Along with these results, experimental studies demonstrated that the transgenic renal proximal tubular epithelial expression of MMP2 was able to generate the entire spectrum of pathological and functional changes characteristic of human chronic kidney disease [17]. Interstitial fibrosis may also be the consequence of various degrees of activation and expansion of the intrinsic fibroblast population or infiltration by mesenchymal stem cells. Several authors have emphasized the potential role of epithelial-mesenchymal transition (EMT) in the development of tubular atrophy and interstitial fibrosis [18].

Significant improvements in life expectancy of kidney transplanted patients due to advances in surgery and immunosuppression have been achieved. However, IF/TA remains a daunting problem. Undoubtedly, the reliance on clinical features commonly results in the late identification of IF/TA [19]. Moreover, the complex network of cellular mechanisms in both, graft and peripheral immune compartments, plus nonimmunological factors contributing to graft injury complicates the noninvasive diagnosis of IF/TA, which still requires biopsy histology. At present, it is a great challenge to identify minimally invasive biomarkers for IF/TA to serve as early predictors of graft loss and as metrics for managing long-term immunosuppression [20].

Based on the key role of ECM turnover in the outcome of IF/TA and the involvement of MMP2 and 9 in normal renal development and glomerular diseases [9], the aim of our study was to investigate the intragraft MMP2 and MMP9 activities and their correlation with renal dysfunction in kidney graft recipients. Additionally, plasma activity of MMP2 and MMP9 was analyzed as an eventual noninvasive biomarker of renal allograft deterioration.

Patients and methods

Patients and biopsies for histopathological classification

Forty-six kidney transplanted patients from Córdoba Hospital (Córdoba, Argentina) were included in a randomized observational cohort study between November 2008 and July 2009. The primary disease represented in transplanted patients was diabetic nephropathy, hypertensive nephropathy, and glomerulopathies. Kidney allograft biopsies were obtained by ultrasound-guided percutaneous puncture at 1, 6, 12 months and once a year onward after transplantation (outlined by protocol) or by clinical reasons at the time of an eventual rejection episode. About two-thirds of each sample was processed for histopathology, and the remaining tissue was frozen at -70°C until use. Histological diagnosis was performed based on tissue studies using light microscopy. Biopsy classification based on Banff '05

criteria [1] resulted in IF/TA negative (IF/TA⁻) and IF/TA positive (IF/TA⁺) samples. Twenty-three IF/TA⁻ and 23 IF/TA⁺ samples were included for the following studies. The IF/TA⁺ cases were graded, in a double-blinded manner, based on severity of tubulo-interstitial features into IF/TA I, IF/TA II, and IF/TA III.

All the transplanted patients received similar immunosuppressive therapy with cyclosporine (doses to reach the following (C2) levels: 1000 ng/ml in the first month post-transplantation, 800 ng/ml until 3rd month, 600 ng/ml until 12th month, and 400 ng/ml thereafter), mycophenolate, and prednisone.

Age, gender, and time post-transplantation (further grouped as <1 year and ≥1 year post-transplantation) were recorded at the time of blood, urine, and kidney biopsy collection.

The study protocol was approved by the Ethics Committee from Córdoba Hospital. Samples were collected after informed consent from the patients or their relatives.

Specimen collection and tissue preparation

Blood and urine samples were simultaneously collected with respective kidney biopsy. Blood samples were collected into potassium-EDTA (at 4 °C) and silica gel-coated plastic tubes for plasma and serum collection, respectively. Plasma samples were immediately frozen at -70 °C for MMPs analysis. Serum samples were used for creatinine determinations. Plasma samples from 14 healthy volunteer blood donors were collected serving as age- and sex-matched reference group.

Isolated urine samples were collected for the determination of urinary protein/creatinine ratio (P/C ratio). Kidney specimens (approximately 5 mg), stored at -70 °C, were homogenized for the zymographic studies. Briefly, tissue samples, randomly selected in accordance to histopathological characterization, were rinsed with phosphate buffer saline plus 1 mmol/l phenylmethylsulphonyl fluoride (PMSF) and homogenized with a glass-Teflon homogenizer in 10% v/v glycerol-RIPA buffer plus 1 mmol/l PMSF, 50 μmol/l leupeptin, and 0.02 mg/ml aprotinin. Protein concentrations were determined using the Bradford protein assay.

Determination of serum and urinary creatinine and urinary protein

Serum and urinary creatinine were measured by the modified Jaffe's method [21] using an automated analyzer. The Modification of Diet in Renal Disease (MDRD) equation was used as a prediction of glomerular filtration rate (GFR) [22].

Urinary protein was checked by dipstick urinalysis (Multistix 10 SG; Siemens Healthcare Diagnostics Inc., Malvern, PA, USA), and afterwards it was quantified using the sulfosalicylic acid test. Urinary P/C ratio was determined. Values higher than 0.2 were considered markers of renal dysfunction [23].

Gelatin zymography

Gelatinolytic activities of MMP2 and MMP9 were detected by gelatin zymography. Briefly, plasma (2 μl) and tissue samples (40 μg) were analyzed by electrophoresis under nonreducing conditions on 7.5% SDS-polyacrylamide gels containing copolymerized gelatin (1.5% w/v; Sigma Chemicals Co., Saint Louis, MO, USA). After electrophoresis, the gels were rinsed twice with 2.5% v/v Triton X-100 (Sigma) for 90 min and incubated 40 h at 37 °C in 50 mmol/l Tris-HCl pH 7.4, containing 9 mmol/l CaCl₂ and 200 mmol/l NaCl. The gels were stained in 45% v/v methanol-5% v/v glacial acetic acid containing 0.125% w/v Coomassie Brilliant Blue R250 (Sigma) and destained in 25% v/v ethanol-10% v/v glacial acetic acid. MMPs activities were detected as clear bands on the blue background of the stained gel. A capillary blood sample was used as positive control. A pool of normal plasmas was included to normalize the signal and to allow comparisons between gels. Prestained SDS-PAGE Standard Molecular Weight (Bio-Rad, Bio-Rad Laboratories Inc., Munich, Germany) was used to determine the molecular weight of the bands. The GELPRO 3.1 software (Media Cybernetics Inc., Rockville, MD, USA) was used for the analysis of the bands.

Statistical analysis

Values were expressed as mean ± standard deviation (SD) or median. Statistical significance of differences between means was determined by ANOVA test and unpaired *t*-test. *P* ≤ 0.05 was considered to indicate a statistically significant difference. Fisher's exact test was used to analyze statistically significant association between IF/TA development, P/C ratio, and time post-transplantation. Pearson's correlation analysis was used to examine bivariate relationships.

Results

Histological analysis of renal allograft biopsies from graft recipients

Adequate renal allograft biopsies were identified from 46 graft recipients. Basic biochemical and histopathological findings of patients' samples are summarized in Table 1. A significant association between IF/TA development and time post-transplantation could be determined.

Table 1. Characteristics of the transplanted groups under study.

	Transplanted IF/TA+	Transplanted IF/TA-	P
Age (years)*	37.8 ± 14.6	34.9 ± 14.9	ns
Gender†			
Male	13	15	ns
Female	10	8	ns
Creatininemia (mg/dl)*	2.12 ± 1.69	1.21 ± 0.29	≤0.05§
Glomerular filtration rate (GFR) (ml/min/1.73 m ²)*	45.0 ± 27.9	65.3 ± 20.1	≤0.05§
Urine Protein/Creatinine ratio†,‡			
>0.2	10	3	
≤0.2	12	20	≤0.05¶
Time post-transplantation†			
<1 year	3	16	≤0.05¶
≥1 year	20	7	
Histological Banff grade†			
I	12	–	
II	10	–	
III	1	–	
Glomerular sclerosis †			
Absence	9	20	≤0.05¶
Presence	14	3	
Mononuclear infiltration†			
Negative	1	9	≤0.05¶
Mild	11	14	
Moderate/High	11	0	
Tubulitis†			
Negative	11	20	≤0.05¶
Mild	7	3	
Moderate/High	5	0	

IF/TA, interstitial fibrosis and tubular atrophy; ns, no significant.

Mononuclear infiltration: Negative, no or trivial inflammation (<10% of unscarred parenchyma); Mild, 10–25% of parenchyma inflamed; Moderate, 26–50% of parenchyma inflamed; High, more than 50% of parenchyma inflamed.

*Mean ± SD is represented.

†Number of cases is indicated.

‡One case could not be determined.

§Unpaired t-test.

¶Fisher's exact test.

The biochemical markers of renal function reflected a moderate level of renal impairment in IF/TA+ patients. Conventional histologic analysis of diagnostic biopsies from this group of patients was carried out. A significant association between glomerular sclerosis, the presence of tubulitis, and IF/TA development was observed ($P \leq 0.05$, Fisher's exact test). Besides, 22 out of 23 IF/TA+ samples showed mononuclear infiltration with a significant association between moderate and high infiltration and IF/TA development ($P \leq 0.05$, Fisher's exact test). On the other hand, only a light infiltration was detected in 14 out of 23 IF/TA- samples.

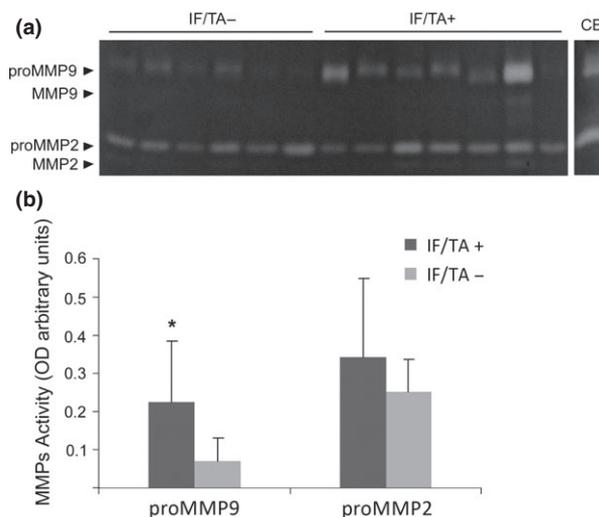


Figure 1 ProMMP2, MMP2, proMMP9, and MMP9 activities in kidney samples. (a) Representative gelatin zymography of kidney samples from transplanted patients (IF/TA+ and IF/TA-). CB, Capillary blood sample. (b) Densitometric analysis of the metalloproteinase activity bands indicates a significant increase in proMMP9 levels in IF/TA+ compared to IF/TA- patients ($*P \leq 0.05$, Student's *t*-test). No significant differences were observed in proMMP2 levels between both groups. Results are expressed as mean ± SD. IF/TA, interstitial fibrosis and tubular atrophy.

No significant differences were observed in the proportion of diabetic IF/TA+ and diabetic IF/TA- patients (21.7% vs. 13.0%, respectively).

Gelatin zymographic analysis for kidney MMP2 and MMP9

Gelatin zymograms were used to determine the MMP2 and MMP9 gelatinolytic activities in kidney samples from transplanted patients. This method allows the identification of areas of protease activity by clear bands for proMMP9 (97 kDa), active MMP9 (86 kDa), proMMP2 (72 kDa), and active MMP2 (66 kDa).

As shown in Fig. 1a,b, a significant increase ($P \leq 0.05$) in gelatinolytic activity of proMMP9 was observed in IF/TA+ in relation to IF/TA- patients. When MMP2 activity was analyzed, an increase in proMMP2 activity was also detected in IF/TA+ patients compared with those IF/TA-, but this failed to reach statistical significance. The active form of MMP2 was only observed in few IF/TA+ patients (2 out of 11 of the samples analyzed).

Gelatin zymographic analysis for plasmatic MMP2 and MMP9

Circulating activities of the proenzymes MMP2 and MMP9 in healthy subjects and transplanted patients were assayed

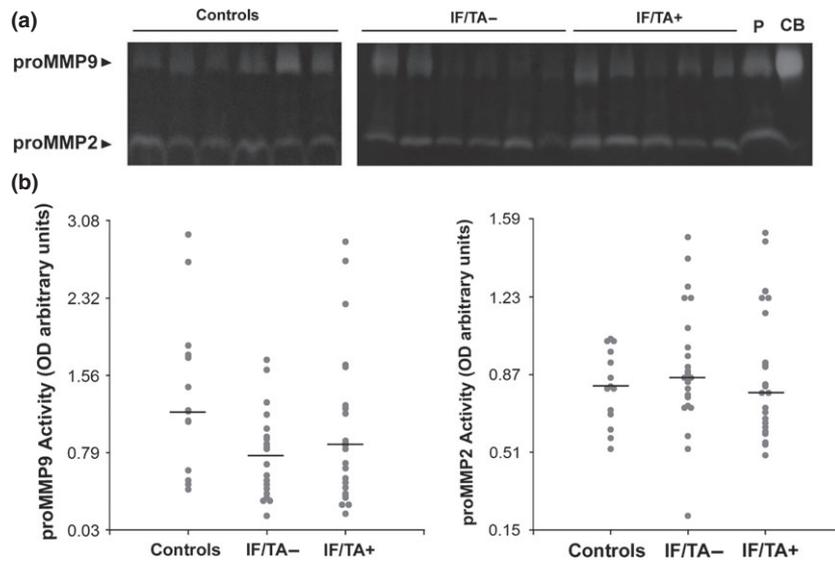


Figure 2 ProMMP2 and proMMP9 activities in plasma samples. (a) Representative gelatin zymography of plasma samples demonstrating proMMP2 and proMMP9 gelatinolytic activities in control individuals, IF/TA- and IF/TA+ transplanted patients. P, Pooled normal plasma sample; CB, Capillary blood sample. (b) Densitometric analysis of the prometalloproteinase activity bands indicates no significant differences between the groups (Kruskal-Wallis Test). The medians are indicated in the figure. IF/TA, interstitial fibrosis and tubular atrophy.

by gelatin zymography. As shown in Fig. 2, circulating proMMP2 and proMMP9 activity in transplanted patients did not show significant differences compared with control samples. Moreover, no significant differences were detected in proMMP2 and proMMP9 gelatinolytic activities between IF/TA+ and IF/TA- groups.

Correlation between kidney gelatinolytic activity, plasmatic MMPs, and GFR in transplanted patients

Kidney proMMP9 and proMMP2 activity and GFR were correlated to check for an eventual association between gelatinolytic activity and renal dysfunction in transplanted patients. A significant inverse correlation was found between kidney proMMP9 and GFR (Fig 3a, Pearson’s test, $P \leq 0.05$, $r = -0.54$). Instead, no significant correlation was observed between GFR and kidney proMMP2 activity (Fig. 3b). When plasma proMMP9 and proMMP2 activities were correlated with the GFR, no significant correlation was detected (data not shown).

Plasma and kidney proMMPs activity relationship was analyzed to assess proMMPs plasmatic activity as a reflection of tissue enzymatic activity. However, no significant correlation was observed between plasma and kidney proMMPs activities in transplanted patients (data not shown).

Discussion

Fibrosis involves an excessive accumulation of ECM and usually results in loss of the organ function. Diffuse inter-

stitial fibrosis, as that observed in the histological analysis of chronically rejected human renal allografts, strongly correlates with impaired graft function [24]. In agreement with these authors, the cohort of IF/TA+ patients involved in our study, where established structural injury was present, showed a significant increase in serum creatinine and P/C ratio together with a significant decrease in GFR. In addition, a significant association between IF/TA development and time post-transplantation could be determined.

MMP2 and MMP9 play a role in both interstitial matrix turnover (via gelatinolytic activity) and basement membrane turnover (via collagen IV and V breakdown). To better understand IF/TA development, we studied MMPs enzyme activity in kidney and plasma samples in relation to the histological findings and renal function. Kidney biopsies from transplanted patients mainly showed the expression of proMMP2 and proMMP9. When renal biopsies were histopathologically classified as IF/TA+ or IF/TA-, proMMP9 showed a significant increase in IF/TA+ samples. In addition, the significant correlation obtained between kidney proMMP9 and the GFR, a basic hallmark of renal dysfunction, suggests the involvement of MMPs in IF/TA development. In accordance with our results, an increased MMP9 expression in kidney allografts has been described as an early event in chronic allograft nephropathy in rats [12,25], in tight correlation with kidney mononuclear infiltration and TGFβ1 expression in the tubulo-interstitium [25].

In the present study, we also described a significant increase in mononuclear cell infiltration in IF/TA+ biop-

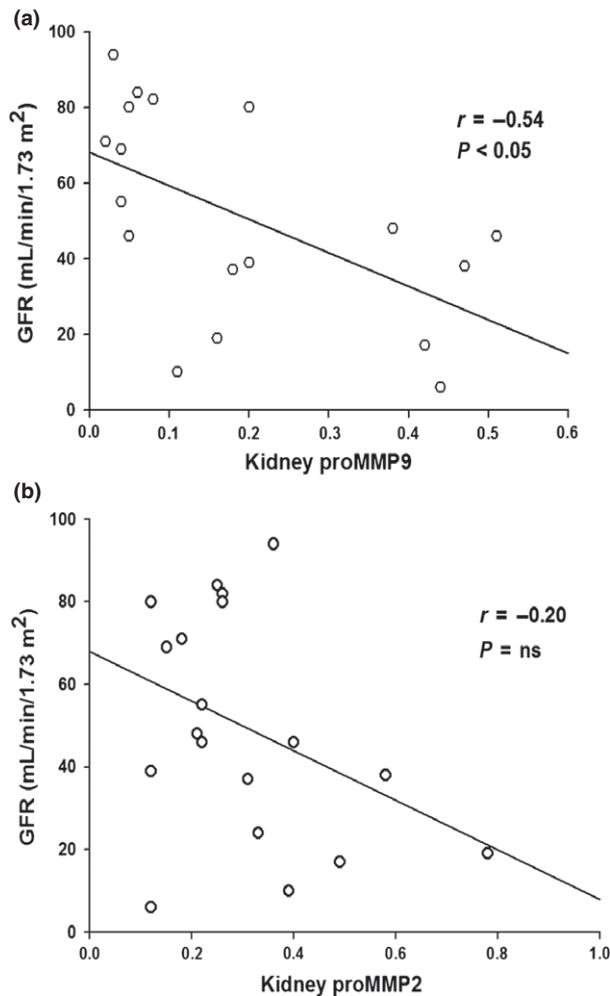


Figure 3 (a) Significant linear correlation between kidney proMMP9 activity and glomerular filtration rate (GFR) in transplanted patients (Pearson's test, $P \leq 0.05$). (b) No significant correlation between kidney proMMP2 and GFR in transplanted patients (Pearson's test).

sies, with the presence of a mild and moderate/high mononuclear infiltration in 95.6% of IF/TA+ samples (22 out of 23). Lymphocytes and macrophages could contribute to those features observed in IF/TA+ biopsies. For instance, MMP9 is a major secretion product of macrophages, lymphocytes, and fibroblasts. Recent studies conducted with a kidney tubular cell line provided evidence that MMP9 secreted by effector macrophages can induce tubular cell EMT, nowadays hypothesized to be related with the pathogenesis of chronic renal allograft rejection [26,27]. On the other hand, it is largely known that MMP9 is involved in inflammatory processes and that both MMP9 and MMP2 are required for T lymphocyte migration into tissues [28]. In relation with this, Robertson and coworkers [29] suggested that TGF β secreted by infiltrating T cells induces the transformation of adjacent tubular epithelial cells in proliferating fibroblasts that migrate across the tubular basement

membrane and participate in the fibrotic process. Other studies have proved the role of MMP2 in the structural alterations in kidney tubular basement membrane, which has been related with EMT and the resultant tubular atrophy, fibrosis, and renal failure [17,27].

MMP2 and MMP9 appeared as constitutively expressed enzymes during renal development, and pleiotropic effects of MMP2 and MMP9, which can account for a protective or detrimental impact in renal disease, have been demonstrated [30]. MMPs regulate cell behavior in addition to degrade ECM components [31]. Therefore, the presence of MMP 2 may have another role in IF/TA development than ECM turnover.

Several studies have demonstrated the participation of glomerular mesangial cells in both the acute inflammatory and chronic sclerotic processes characteristic for many forms of glomerular diseases. Human mesangial cells can secrete MMP2 and MMP9. A close linkage between mesangial cell activation (augmented proliferation and synthesis of ECM proteins) and high-level synthesis of MMP2 that directly mediates the evolution of the sclerotic state has been demonstrated [32]. In our study, kidney proMMP2 in IF/TA+ patients tended to increase compared with IF/TA- patients, and this finding may be related with the expansion of glomerular mesangial cells observed in some of the biopsies (60%; 14 out of 23 biopsies).

As was previously mentioned, MMPs regulate cell behavior through tightly controlled proteolytic processing of signaling molecules, growth factors, membrane receptors, and chemokines [31]. MMPs can also modulate the net proteolytic potential through modulation of the activation states of different interconnected proteases, being the cause of unexpected pleiotropic effects derived from alteration of other proteolytic pathways. Regarding to ECM synthesis, MMP2 and MMP9 have been associated with the activation of TGF β through cleavage of latency-associated peptide (LAP) [33]. This activated growth factor has the possibility to repress MMPs activation which in turn could promote connective tissue growth factor (CTGF) anabolic pathways leading to ECM synthesis and accumulation. Our results showed an increased proMMP9 activity associated with interstitial fibrosis suggesting alternative or additional mechanisms than mere matrix degradation in the control of ECM homeostasis.

In agreement with our results, experimental studies inhibiting MMP2 and MMP9 activities early after kidney transplantation reduced the development and progression of CAN in rats [34].

In the present study, we also analyzed MMP2 and MMP9 plasma activities in transplanted patients and control subjects. No significant differences among IF/TA+, IF/TA-, and nontransplanted subjects' plasma proMMP2 and

proMMP9 activities were observed. Besides, when plasma and kidney MMPs activities were compared in transplanted patients, neither plasma proMMP2 nor plasma proMMP9 correlated with kidney proMMP2 and proMMP9 activities. In many pathological conditions, plasma MMP2 and MMP9 have been used to reflect important tissular damage [35,36]. In kidney pathologies, MMPs play a main role [9]; however, circulating profile of MMP2 and MMP9 showed varied results suggesting the involvement of different mechanisms in the regulation of tubulo-interstitial fibrosis in renal diseases [37]. In kidney transplantation, inconsistent results have been published, depending on methodology or clinical status of the patients. A high serum MMP2 was described in CAN patients [13], even though other authors could not demonstrate an increased plasma MMP2 [38] or a high intragraft MMP2 in kidney biopsies [16]. Recently, an increased plasma MMP9 has been described in kidney transplanted patients with decreased GFR; however, an stable MMP9:TIMP1 index was observed in kidney recipients and normal subjects [38]. In our study, IF/TA+ patients showed a high kidney MMP9 although no plasma MMP9 increase could be reported.

The data presented here provide evidence that relates MMPs with the fibrotic changes of IF/TA. The accumulation of ECM proteins may be explained by a dysregulation of MMPs, but the elevation of proMMP9 as well as the presence of proMMP2 in the kidney in IF/TA+ group could be reflecting a local event pointing at ECM turnover increase, a decrease in gelatinase clearance or in the activity of TIMPs over these molecules. Thus, further studies aiming at clarifying the role of gelatinases in IF/TA are needed, not only to understand the pathogenic mechanism of renal fibrosis and glomerulosclerosis, but also to help to the early detection of chronic graft rejection and to improve graft survival.

Authorship

MAR: participated in performing the study. MAR, ACD: drafting and revision of the manuscript. PAN, IR, MD, ACD: contributed to the conception of the work. Contributed with important reagents and data collection. Revising the manuscript critically for important intellectual content. MAR, ABDV, CGP, MD, ACD: contributed to the acquisition, analysis, and interpretation of data. CGP: contributed with important reagents and the revision of the manuscript.

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