Dysregulation of immunoexpression of matrix metalloproteinases in renal chronic allograft injury

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The aim of the study was to evaluate tubulointerstitial immunoexpression of MMPs and TIMPs in chronic renal allograft injury, and to assess any relationships between immunoexpression of MMPs and interstitial monocytes/macrophages and mast cells. Immunohistochemistry with antibodies against MMP-2, MMP-2, MMP-12, TIMP-1, TIMP-2, CD68, and mast cells tryptase was carried out on 17 renal biopsy specimens from patients with chronic allograft injury, and on 11 control kidney tissues. In renal specimens in chronic allograft injury increased expression of MMP-2, MMP-12 and TIMP-1 was noted. The immunoexpression of MMP-9 and TIMP-2 was low, and did not differ in comparison with controls. Increased immunoexpression of MMP-12 was positively correlated with the number of interstitial CD68+ cells. The correlation between immunostaining of MMP-12 and mast cells tended to be positive, however it did not reach statistical significance. There were significant positive correlations between immunostaining of MMP-9 and CD68+ cells, as well as between MMP-9 and the number of mast cells. In conclusion our study revealed that the remodelling of kidney structure in patients with chronic allograft injury is associated with dysregulation of MMPs and TIMPs, and may suggest that interstitial monocytes/macrophages and mast cells may cooperate with MMPs in pathogenesis of renal fibrosis.

Key words: renal allograft, matrix metalloproteinases, renal fibrosis, immunohistochemistry

Introduction

Chronic renal allograft injury is characterized by interstitial fibrosis, tubular atrophy, vascular lesions, glomerulosclerosis and slowly progressive functional impairment. Since the 8th Banff conference the term chronic graft injury with no evidence of known aetiology has been defined and three grades according to the severity and extent of damage have been proposed [1]. It is documented that a severe grade of renal damage with extensive interstitial fibrosis, tubular atrophy, arteriolar hyalinosis, and a high percentage of glomerulosclerosis has an adverse influence on graft prognosis [2]. Despite the undoubted significance of renal interstitial fibrosis in chronic allograft damage, the complex, multistage mechanisms that result in this process are poorly understood. Renal fibrosis manifests as the accumulation of extracellular matrix (ECM) that can result from increased synthesis or decreased degradation of ECM as well as a combination of both [3, 4]. Matrix metalloproteinases (MMPs) are a family of more than 20 secreted or transmembrane proteins that are capable of digesting extracellular matrix and basement membrane components under physiological conditions. According to their substrate specificity and structure, MMPs are classified into six subgroups: collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), metalloelastase (MMP-12), membrane-type MMPs (MMP14, MMP15), and other MMPS (e.g., MMP-19 and MMP20). MMP-2 (gelatinase A) predominantly degrades fibronectin and laminin, whereas MMP-9 (gelatinase

B) degrades collagen type IV and V [5]. Human macrophage elastase (HME; MMP-12) was first identified as an elastolytic metalloproteinase secreted by inflammatory macrophages 30 years ago [6]. MMP-12 shares many features typical of MMPs, including its domain structure, chromosomal location within the MMP gene cluster on human chromosome 11q22, and capacity to degrade extracellular matrix components [7]. In addition to elastolytic activity, MMP-12 has been shown to be capable of degrading a broad spectrum of other extracellular matrix components, including type IV collagen, fibronectin, laminin, vitronectin, proteoglycans, chondroitin sulfate, and myelin basic protein. It is well known that activities of MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) through formation of non-covalent complexes with MMPs. The tissue inhibitor of metalloproteinases comprises a four-member family of homologous MMP inhibitors (TIMP-1, -2, -3, -4) [8]. The transcription of TIMPs is regulated by similar cytokines and growth factors that control MMPs expression, although often in distinctive ways. On the other hand, experimental models and biopsy evidence have demonstrated the presence and importance of macrophages and T cells in chronic allograft injury and the generation of fibrosis [9]. Profibrotic cytokines released by macrophages induce a phenotypic switch in the effector cells resulting in ECM accumulation [10]. Moreover, several studies have shown that the number of interstitial mast cells increases in acute renal allograft rejection and correlates well with the magnitude of interstitial fibrosis [11, 12]. These findings suggest that mast cell tryptase plays a role in the proliferation and extracellular matrix protein production of renal interstitial fibroblasts and contributes to the development of renal interstitial fibrosis [13]. Thus the present study was undertaken to evaluate tubulointerstitial immunoexpression of MMP-2, MMP-9, MMP-12, TIMP-1 and TIMP-2 in chronic renal allograft injury, and to assess any relationships between immunoexpression of MMPs and interstitial monocytes/macrophages and mast cells.

Material and methods

Patients

Seventeen renal biopsy tissues from patients with chronic allograft injury with no evidence of known aetiology were collected from the archive of the Department of Nephrology. Biopsies were performed 13-35 months after kidney transplantation (mean 21 ±11 months). The kidneys were from cadaver donors. All the patients were adults: the mean age was 42.3 ±9.1 and the male to female ratio was 11 : 6. Patients' serum creatinine levels ranged from 2.9 mg/dl to 5.5 mg/dl (mean 2.9 ±0.9 mg/dl). Proteinuria was < 500 mg/24 h in all patients. Morphological diagnosis of chronic graft injury was established independently by two experienced nephropathologists; it was based on light microscopy, immunofluorescence and electron microscopy, and scored according to the Banff definitions [14]. In all cases mild interstitial fibrosis and tubular atrophy (< 25% of cortical area) with no evidence of any specific aetiology (Grade I Banff '05) were seen. Immunosuppression consisted of either a cyclosporine-based or tacrolimus-based regimen. As a control 11 allograft biopsy specimens from patients without any signs of rejection were used (the male to female ratio was 8 : 3, the mean age was 41.9 ±9.3.

Immunohistochemistry

Paraffin sections were mounted onto superfrost slides, deparaffinized, then treated in a microwave oven in a solution of 1 mM EDTA, pH 8.0 for 20 min and transferred to distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in distilled water for 5 minutes, and then sections were rinsed with Tris-buffered saline (TBS, Dako, Denmark) and incubated with: monoclonal mouse-anti-human matrix metalloproteinase-2 (clone 17B11, Novocastra Lab., dilution 1 : 50), monoclonal mouse anti-human matrix metalloproteinase-9 (clone 15W2, Novocastra Lab., dilution 1 : 100), monoclonal mouse-anti-human tissue inhibitor of matrix metalloproteinase-1 (clone 6F6a, Novocastra Lab., dilution 1 : 75), monoclonal mouse anti-human tissue inhibitor of matrix metalloproteinase-2 (clone 46E5, Novocastra Lab., dilution 1 : 50), rabbit monoclonal anti-human MMP-12 antibody (EP1261Y- carboxy-terminal end, Abcam, dilution 1 : 100), monoclonal mouse anti-human mast cell tryptase antibody (clone: AA1, Dako, Denmark, dilution 1 : 100), and monoclonal mouse anti-human CD68 antibody (Dako, Denmark, dilution 1 : 100). Afterwards LSAB+/HRP Universal kit (Dako, Denmark) prepared according to the instructions of the manufacturer was used. Visualisation was performed by incubating the sections in a solution of 0.5 mg 3,3'-diaminobenzidine (Dako, Denmark), per ml Tris-HCl buffer, pH 7.6, containing 0.02% hydrogen peroxide, for 10 minutes. After washing, the sections were counter-stained with haematoxylin and coverslipped. For each antibody and for each sample a negative control was processed. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

In each specimen staining intensity of MMP-2, MMP-9, MMP-12, TIMP-1 and TIMP-2 in the tubulointerstitium were recorded semiquantitatively by two independent observers in 7-10 adjacent high power fields and graded 0 (staining not detectable), 1 (minimal immunostaining in some cells), 2 (weak immunostaining intensity in all cells) or 3 (strong staining in all cells). The mean grade was calculated by averaging grades assigned by the two authors and approximating the arithmetic mean to the nearest unity.

Histological morphometry was performed by means of an image analysis system consisting of a PC equipped with a Pentagram graphics tablet, Indeo Fast card (frame grabber, true-colour, real-time), produced by Indeo (Taiwan), and Panasonic colour TV camera (Japan) linked to a Carl Zeiss microscope (Germany). This system was programmed (Multi-Scan 8.08 software, produced by Computer Scanning Systems, Poland) to calculate the number of objects (semi-automatic function) and the surface area of a structure using a stereological net (with regulated number of points). The coloured microscopic images were saved serially in the memory of a computer, and then quantitative examinations were carried out. Interstitial monocytes/macrophages and mast cells were determined by counting CD68+ cells and cell tryptase positive mast cells (semi-automatic function) in a sequence of ten consecutive computer images of 400 × high power fields – 0.0047 mm^2 each. The only adjustments of field were made to avoid glomeruli and large vessels. The results were expressed as a mean number of CD68 and mast cell tryptase immunopositive cells per mm^2.

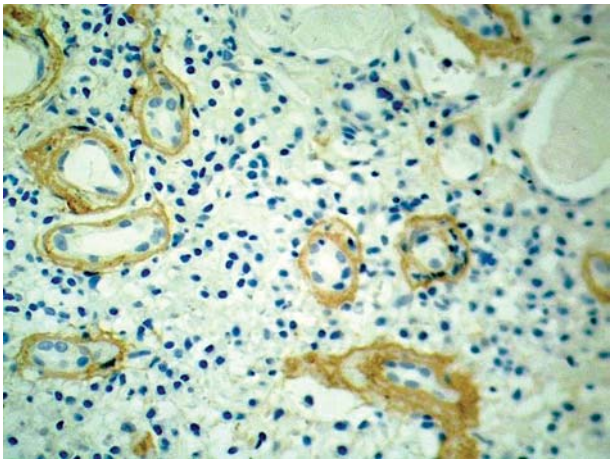

Fig. 1. Moderate focal immunoexpression of MMP-2 in renal tissue in patient with chronic allograft injury. Magnification 400 ×

Statistical methods

All values were expressed as the mean ± SD (standard deviation). The differences between groups were tested using Student's t-test for independent samples preceded by evaluation of normality and homogeneity of variances with Levene's test. Additionally the Mann-Whitney U test was used where appropriate. Correlation coefficients were calculated using Spearman's method. Results were considered statistically significant if $p < 0.05$.

Results

In renal biopsy specimens in the control group only very slight focal expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in the interstitium and tubular epithelial cells was seen. Our study did not reveal immunoexpression of MMP-12 in kidney tissues in controls. The semi-quantitative data of the immunoexpression of MMP-2, MMP-9, MMP-12, TIMP-1, TIMP-2, CD68+ cells and tryptase+ mast cells appear in Table I. The immunohistochemical staining in renal biopsy specimens in chronic allograft injury showed increased expression of MMP-2 (Fig. 1), MMP-12 and TIMP-1 (Fig. 2); however, immunoexpression of MMP-2 did not reach statistical significance ($p = 0.7$). The immunoexpression of MMP-12 was seen within areas of inflammatory infiltrates (Fig. 3). The immunoexpression of MMP-9 and TIMP-2 were low in the chronic allograft disease group, and did not differ in comparison with controls. The mean numbers of interstitial CD68+ cells and mast cells were in

Table I. Tubulointerstitial expression of MMP-2, MMP-9, MMP-12, TIMP-1, TIMP-2, the number of interstitial CD68+cells and the number of tryptase+ mast cells in renal biopsy specimens in chronic allograft injury (CAI) and controls

Number of cases	MMP-2 (mean score)	MMP-9 (mean score)	MMP-12 (mean score)	TIMP-1 (mean score)	TIMP-2 (mean score)	Tryptase+ (cells/mm^2)	CD68+ (cells/mm^2)
Controls (n = 11)	1.1 ±0.46	1.85 ±0.78	1.63 ±0.59	0.35 ±0.18	0.42 ±0.12	1.35 ±0.89	48.78 ±20.67
CAI (n = 17)	1.48 ±0.56	1.51 ±0.66	2.81 ±0.87	0.71 ±0.53	0.53 ±0.31	5.26 ±2.97	92.33 ±37.21
p value	0.07 (NS)	0.22 (NS)	< 0.001	< 0.05	0.22 (NS)	< 0.001	< 0.002

Data are expressed as mean values ±SD
NS – not significant

renal tissues from patients with chronic allograft lesions significantly increased ($p < 0.002$, and $p < 0.001$, respectively). In the chronic allograft injury group there were significant positive correlations between immunostaining of MMP-9 and CD68+ cells, as well as between MMP-9 and the number of mast cells. We did not find a significant relationship between MMP-2 and CD68+ cells and the number of mast cells. In chronic allograft injury increased immunoexpression of MMP-12 was positively correlated with the number of interstitial CD68+ cells. The correlation between immunostaining of MMP-12 and mast cells tended to be positive, but it did not reach statistical significance ($p = 0.6$). In controls all these correlations were not significant. The correlations between the tubulointerstitial immunoexpression of MMPs and CD68+ cells, and tryptase positive mast cells are shown in Table II.

Discussion

A wealth of knowledge has been accumulated to show that MMPs play many roles in both biological and pathological processes, but the literature data regarding the expression of matrix metalloproteinases in allograft injury are controversial [15-18]. The cellular and molecular mechanisms underlying the histopathological lesions in chronic allograft disease remain obscure. Many cell-cell, cell-matrix, and intercellular pathways have been implicated in the complex pathogenesis of chronic allograft nephropathy [18]. Matrix metalloproteinases are critical regulators of matrix deposition, and uncontrolled ECM remodelling of the kidney is a feature of renal disorders. Our study showed dysregulation of immunoexpression of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinase in renal tissue in chronic allograft injury. The immunohistochemical staining in renal biopsy specimens in chronic allograft injury showed increased expression of MMP-2, MMP-12, and TIMP-1; however, increased expression of MMP-2 did not reach statistical significance. The immunoexpression of MMP-9 and TIMP-2 was low in the chronic allograft disease group, and did not differ from controls. In the study of Inkinen *et al*. [15] in rat kidney allografts with fibrosis these proteins were dysregulated with increased expression of MMP-2

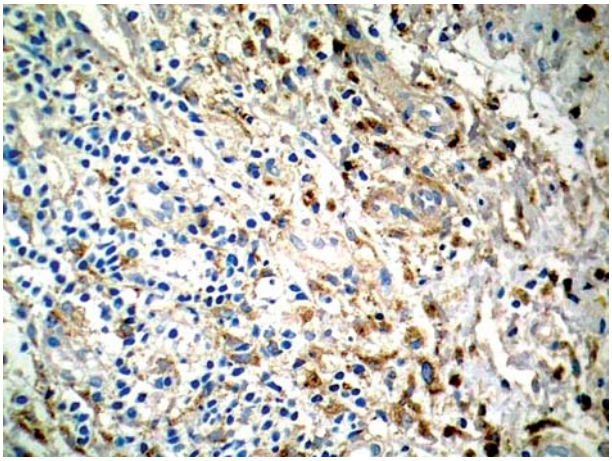

Fig. 2. Strong immunostaining of MMP-12 in renal biopsy specimen in patient with chronic allograft injury. Magnification 400 ×

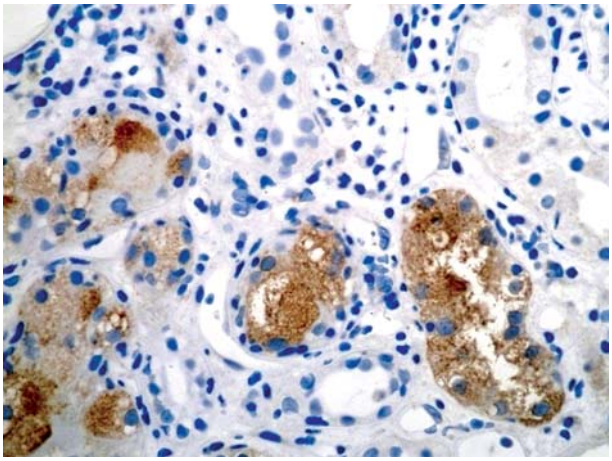

Fig. 3. Moderate and focal immunostaining of TIMP-1 in renal tissue in patients with chronic allograft injury. Magnification 400 ×

Table II. The correlations between tubulointerstitial immunoexpression of MMPs and tryptase positive mast cells, as well as CD68 positive cells in renal tissues in chronic allograft injury (CAI) and controls

Correlation between:	CAI (n = 17)	Controls (n = 11)
immunoexpression of MMP-2 and tryptase+ mast cells	r = 0.38, p = 0.13 (NS)	r = 0.22, p = 0.52 (NS)
immunoexpression of MMP-9 and tryptase+ mast cells	r = 0.52, p < 0.04	r = 0.06, p = 0.86 (NS)
immunoexpression of MMP-12 and tryptase+ mast cells	r = 0.46, p = 0.06 (NS)	r = 0.17, p = 0.61 (NS)
immunoexpression of MMP-2 and CD68+ cells	r = 0.42, p = 0.09 (NS)	r = 0.35, p = 0.29 (NS)
immunoexpression of MMP-9 and CD68+ cells	r = –0.68, p < 0.003	r = 0.36, p = 0.27 (NS)
immunoexpression of MMP-12 and CD68+ cells	r = 0.55, p < 0.05	r = –0.08, p = 0.81 (NS)

NS – not significant

and MMP-9. However, it must be taken into consideration that immunoexpression of MMPs and TIMPs is related to the phases of fibrosis. Lutz *et al*. [16] showed that inhibition of MMP-2, -3 and -9 early post transplant improved proteinuria and histology of kidney, while at the later time point appeared to aggravate the disease. These authors concluded that the results pointed to the pathogenetic time-course-dependent differential role of MMPs in the development and progression of chronic allograft nephropathy, and attention must be paid to the temporal relationship between MMP inhibition and therapeutic outcome. In chronic allograft nephropathy patients, serum MMP-2 and MMP-3 increased [19], while in renal tissues MMP-2 decreased [20]. In an experimental study in chronic renal allograft rejection, mRNA tissue level of MMP-9 was decreased, and mRNA of MMP-2, MMP-12 and TIMP-1 and TIMP-2 were found to be significantly augmented [17]. In the study of Breathier *et al*. [17] MMP-9 decreased substantially during allograft rejection, but Mass *et al*. [18] in human chronic allograft nephropathy found overexpression of MMP-9 in renal samples. Similarly, genes related to fibrosis and extracellular matrix deposition, including the MMP-9 gene, were found up-regulated in chronic allograft nephropathy [18]. The discrepancies between our results and microarray studies may be due to the fact that transplant tissue biopsy samples used for microarray analysis may have contained a mixture of different cell types including inflammatory cells that contribute to the resulting profile signature types [18]. Nicholson *et al*. [21] found that intragraft expression of mRNA for TIMP-1 and TIMP-2 is significantly correlated with human allograft fibrosis and immunoexpression of TGF-β. These authors suggest that alterations in the ratio of TIMPs and MMPs in the transplant kidney may be an important molecular mechanism leading to the development of tubulointerstitial fibrosis. The abundance of TIMP-1 in kidneys was increased significantly in most experimental models and several human renal diseases, and the degree of TIMP-1 increase was associated with the extent of fibrosis [22]. Cai *et al*. [23] found that overexpression of TIMP-1 could promote renal interstitial fibrosis through an inflammatory pathway, which may be partly induced by upregulating ICAM-1. The pathogenesis of renal fibrosis during chronic allograft disease is complex, multifactorial, and incompletely understood. It is worth noting that the excess ECM deposited is of a different composition to that normally found in the kidney and changes according to the duration of chronic allograft nephropathy [24]. The deposition of ECM after decreased MMP expression in progressive fibrosis may disrupt the interaction between tubular epithelial cells and interstitial fibroblasts. In these cases ECM deposition may become self-perpetuating because collagen IV interacts with growth factors such as platelet-derived growth factor (PDGF), thus stimulating the proliferation of interstitial fibroblasts and leading to progressive tissue injury [25]. In our study immunoexpression of MMP-12 showed the strongest increase in biopsy tissues in chronic renal allograft injury. Metalloelastase (MMP-12) digests elastin and the number of ECM molecules and is expressed primarily in macrophages. The role of MMP-12 in allograft rejection remains to be defined, but this matrix metalloproteinase previously was shown to play a role in inflammation [26]. One apparently important function of catalytic MMP-12 *in vivo* is its ability to activate other MMPs such as MMP-2 and MMP-3, by which MMP-12 exaggerates the cascade of proteolytic processes [27]. Our findings indicated that immunoexpression of MMP-12 may represent a marker for chronic renal allograft injury. It is noteworthy that immunoexpression of MMP-9 and MMP-12 was correlated with the number of interstitial monocytes/macrophages and mast cells. The presented data suggest that alteration in the expression of MMPs in the transplant kidney may be an important mechanism leading to the development of interstitial fibrosis. Moreover, the correlation between MMPs and CD68+ cells and tryptase+ cells supports the role of monocytes/macrophages and mast cells in the development of chronic renal allograft injury. Inflammatory cells in a renal graft consist of T lymphocytes and monocytes/macrophages. It is well known that macrophages secrete a host of profibrotic cytokines: transforming growth factor β (TGF-β), tumour necrosis factor α (TNF-α), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and matrix proteins. Mast cells can activate fibroblasts and promote collagen synthesis, and have been shown to produce cytokines and growth factors that may contribute to fibrosis, whereas heparin and tryptase may enhance fibroblast migration and proliferation [28]. In the kidney, macrophage-derived TGF-β may promote fibrosis by paracrine activation of matrix-producing myofibroblasts and promotion of tubular epithelial cell differentiation into myofibroblasts [29].

In conclusion, our study revealed that the remodelling of kidney structure in patients with chronic allograft injury is associated with dysregulation of MMPs and TIMPs, and may suggest that interstitial monocytes/macrophages and mast cells may cooperate with MMPs in pathogenesis of renal fibrosis.

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