

Metzincins, including matrix metalloproteinases and meprin, in kidney transplantation

Céline Berthier, Hans-Peter Marti

Department of Nephrology and Hypertension, Inselspital Berne, University of Berne, Berne, Switzerland

Summary

Chronic allograft nephropathy, including chronic rejection, remains one of the major causes of renal allograft failure. Amongst other mediators, metzincins, such as matrix metalloproteinases (MMP), direct extracellular matrix metabolism and cell proliferation. Thus, we hypothesized, that these proteolytic enzymes are differentially regulated in chronic renal transplant rejection in rats and in human renal allograft nephropathy. Our studies demonstrated on the experimental level

and in humans an overall up-regulation of MMP, tissue inhibitors of metalloproteinases (TIMP) and related enzymes as a result of rejection processes. Thus, metzincins may represent novel markers and therapeutic targets with respect to renal allograft rejection.

Key words: chronic allograft nephropathy; chronic rejection; extracellular matrix; matrix metalloproteinases (MMP); metzincins; cyclosporine

Introduction

Major causes of late renal allograft failure are chronic allograft nephropathy, including chronic rejection, and patient death with a functioning transplant. Therapy and precise diagnosis of chronic rejection remain difficult. Alterations in the extracellular matrix (ECM) compartment and in the proliferation rates of intrinsic and extrinsic renal cells represent important features in chronic allograft nephropathy.

Extracellular matrix metabolism and cell proliferation are to a significant part controlled by the metzincin superfamily of metallo-endopeptidases, including matrix metalloproteinases (MMP), ADAM, ADAMTS and meprin [1-4].

MMP are traditionally classified into four categories, including interstitial collagenases (MMP-1/-8/-13), gelatinases (MMP-2/-9), stromelysins (MMP-3/-7/-10/11/-12) and membrane-type (MT)-MMP (MMP-14/-15/-16/-17), [1, 3]. MMP inhibition mainly occurs by tissue inhibitors of metalloproteinases (TIMP), most importantly by TIMP-1 and TIMP-2 [1, 3].

ADAM represent a family of cell membrane-anchored proteins containing a disintegrin and metalloprotease domain [4]. The metalloprotease domains induce ectodomain shedding releasing cell-surface proteins. This domain also cleaves and remodels extracellular matrix components. The disintegrin and cysteine-rich domains have adhesive properties.

ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) are extracellular proteins that can bind to matrix components [2]. ADAMTS have been found to cleave proteoglycans and matrix components, to inhibit angiogenesis and to be involved in the blood coagulation homeostasis [2].

Meprin represents a zinc endopeptidase of the astacin enzyme family, produced as a membrane-bound or secreted protein in epithelial cells (including renal tubular cells), in leucocytes and in tumour cells [5, 6]. There are two types of meprin subunits, α and β , which form disulphide-bonded homo- and hetero-oligomers. Meprin cleaves a wide range of peptides and proteins, including ECM components.

To investigate the regulation of metzincins in solid organ transplantation, we have used cDNA microarrays. With respect to kidney transplantation, this approach has already been used successfully in the past [7-12]. Sarwal et al. recently published a seminal paper describing consistent differences among gene expression profiles between acute rejection, drug nephrotoxicity, chronic allograft nephropathy, and normal grafts [11]. Importantly, these authors defined three subtypes of acute rejection with prognostic implications that were characterised by differences in immune activation and cell proliferation rates [11]. Furthermore, a clear association between CD20⁺ B-cell

infiltrates and glucocorticoid resistance as well as graft loss was seen [11]. The literature of chronic allograft nephropathy remains less clear, although a few very interesting studies have identified differentially expressed individual genes or gene expression profiles in chronic allograft nephropathy by microarrays [7–10, 12].

In our studies, we mainly investigated met-

zincins and related genes in experimental kidney transplantation and in human chronic allograft nephropathy. Furthermore, we studied effects of an immunosuppressive agent, cyclosporine A, on MMP production. However, these investigations were performed in experimental heart transplantation due to the availability of the respective tissues.

Experimental studies

Experimental chronic renal allograft rejection [13]

In a preceding investigation, as a result of acute rejection in the stringent DA-to-Lewis rat strain combination, overall MMP activity slightly increased and was successfully inhibited by the MMP inhibitor BB-94 [14]. The MMP inhibitor BB-94 was able to keep proteinuria at a low level, although no visible attenuation of histological damage was observed. Thus, MMP does not appear to play a crucial role in severe acute rejection, probably due to the magnitude and redundancy of up-regulated pro-inflammatory mediators.

Thus, we investigated the regulation of metzincins in the established Fisher (F344) to Lewis (LEW) rat model of chronic renal allograft rejection [13]. LEW recipients of F344 allografts develop acute rejection episodes around day +25 after transplantation, leading to approximately 50% graft loss [15, 16]. The surviving rats demonstrate histopathological and functional characteristics of chronic rejection beyond day +50 after transplantation, without concomitant immunosuppressive treatment.

Our studies were performed in close collaboration with Joosten S.A. et al, Department of Nephrology, Leiden University Medical Centre, Leiden, The Netherlands. Analyses were performed at day 0 (healthy controls) and during periods of chronic rejection at days +60 and +100

following transplantation. Messenger RNA expression was examined by real-time Taqman PCR preceded by Affymetrix® Rat Expression Array 230A GeneChip analyses (Novartis Institutes for BioMedical Research, Basel, Switzerland). Protein expression was studied by zymography, Western blot analyses and immunohistology.

Messenger RNA (mRNA) tissue levels of MMPs (MMP-2/-11/-12/-14), of their inhibitors (TIMP-1/-2), of ADAM-17 and of TGF- β 1 were found to be significantly augmented during chronic renal allograft rejection. The most striking mRNA elevation was demonstrated in the case of MMP-12. TIMP-3 remained unchanged. In contrast, mRNA levels of MMP-9/-24 and meprin α/β were decreased. These results are summarised in table 1.

These quantitative real-time PCR data were in accordance with the data obtained by the less quantitative genechip analysis with the notable exception of MMP-2. Microarrays failed to show an increase of MMP-2, because the Affymetrix probe set did not recognise the splice variant of MMP-2 over-expressed in our rat model.

Western blot analysis also depicted increased production of MMP-12, MMP-14 and TIMP-2 (in the latter two cases as individual proteins and as complexes). MMP-2 activity and immunohistological staining were increased in parallel. MMP-9 protein levels and meprin α/β synthesis and activity were down-regulated significantly.

In conclusion, members of metzincin families (MMP/TIMP, ADAM and meprin) are differentially regulated in chronic renal allograft rejection. Thus, altered pattern of metzincins may represent novel diagnostic markers and possibly may provide novel targets for future therapeutic interventions.

Experimental acute heart allograft rejection [17]

In this still-ongoing study, the prime interest was the analysis of the effect of cyclosporine A on MMP and TIMP expression. These investigations were performed in close collaboration with members of Novartis Pharma AG, Basel, Switzerland; mainly with Friedrich Raulf.

For these analyses, we used a rat model of acute heart allograft rejection, the Dark Agouti-to-Lewis (DA-to-Lewis) heterotopic heart allotransplanta-

Table 1

Real-time TaqMan PCR Analyses of Metzincins
Significant up-regulation (\uparrow) and down-regulation (\downarrow) of mRNA levels of rat metzincins in chronic renal allograft rejection and in acute cardiac allograft rejection versus control samples are depicted.

Metzincins	Chronic renal allograft rejection in rats	Acute cardiac allograft rejection in rats
MMP-2	\uparrow	\uparrow
MMP-7		\uparrow
MMP-8		\uparrow
MMP-9	\downarrow	$\uparrow\uparrow$
MMP-11	\uparrow	\downarrow
MMP-12	$\uparrow\uparrow$	$\uparrow\uparrow$
MMP-14	\uparrow	\uparrow
MMP-24	\downarrow	
TIMP-1	\uparrow	$\uparrow\uparrow$
TIMP-2	\uparrow	\uparrow
TIMP-3	\rightarrow	\uparrow
ADAM-17	\uparrow	
Meprin α and β	\downarrow	

tion, with and without the administration of Sandimmune Neoral® (7.5 mg/kg/d, from the time of transplantation). In this model, untreated allograft recipients develop severe acute rejection 5 days after transplantation [18].

In the following, we report preliminary data, essentially as published in abstract form [17]. Three groups of hearts at day +5 after transplantation are being studied: placebo treated DA allografts (n = 6), Sandimmune Neoral® treated DA allografts (n = 6), healthy native DA hearts (n = 6). The mRNA levels of MMP and TIMP were analysed by real-time TaqMan PCR and by Affymetrix® Rat Expression Array 230A GeneChip® analyses (Functional Genomics Centre, University of Zürich). MMP protein expression and activities were quantified by fluorometry, zymography and Western blot assays.

Histological analyses demonstrated a severe acute rejection of grade III in placebo treated heart allografts. The exposure of Sandimmune Neoral® attenuated acute rejection to grade II in treated hearts.

In allografts of placebo treated animals, mRNA levels of MMP (MMP-2/-7/-8/-9/-12/-14) and TIMP (TIMP-1/-2/-3) were clearly increased, as depicted in table 1. Interestingly, maximal increases in mRNA levels were observed for MMP-9, MMP-12 and TIMP-1. MMP-11 mRNA was decreased. Accordingly, overall MMP activity, including activities of pro-MMP-2 and pro-MMP-9, significantly increased during rejection. Accordingly, MMP-2 immunohistology showed an increased staining in untreated DA allografts.

The exposure of Sandimmune Neoral® demonstrated only a trend towards an attenuation of the increased MMP/TIMP production. The only exception was a clear reduction of MMP-14 at the protein level, compatible with the literature [19].

In summary, gene expression of several key MMP and TIMP were up-regulated during acute rejection in heart allografts. Therapy with Sandimmune Neoral® did not show a clear and consistent effect on MMP/TIMP production. Thus, cyclosporine A did not appear to be a key regulator of metzincin synthesis under these conditions.

Human chronic allograft nephropathy (CAN)

The interpretation of renal biopsies is currently based on three approaches: light microscopy, immunofluorescence and electron microscopy. Thus, quantitative analyses of specific parameters by molecular profiling may improve the diagnostic and prognostic yield of a given transplant biopsy [20, 21].

Metzincins and metzincin substrates (mostly extracellular matrix proteins) represent good candidate genes for such an approach, since they already play a key role in the normal physiology of the renal glomerulus and the tubulo-interstitium.

Furthermore, depending upon the type and stage of a given nephropathy, an augmented or even a decreased metzincin or metzincin substrate expression contributes to structural and functional tissue damage.

In this study, we hypothesised the following: (1) In chronic allograft nephropathy, alterations of the expression and the activity of metzincins and their substrates, such as extracellular matrix proteins, in biopsies (and possibly also in leukocytes and serum) reflect glomerular and tubulo-interstitial damage of the transplanted kidney. (2) Analyses of the expression patterns of these genes help to refine the histological diagnosis, to elucidate pathogenetic mechanisms and to predict disease progression in chronic allograft nephropathy. (3) In the future, modifications of medical patient therapy based on our results will help to ameliorate tissue damage in transplanted kidneys with signs of chronic allograft nephropathy.

This collaborative study was designed and initiated by the principal investigator of this grant proposal (H.P.M.) during a one year INSERM position ("Poste Orange") between 2003 and 2004 at Hôpital Tenon, INSERM U489, Paris, France.

All renal transplant patients at the Services de Néphrologie A and B, Hôpital Tenon, Paris, who underwent renal allograft biopsy, were included in the study after having given informed consent, from February 2003 until September 2004; some were later included until September, 2005. In addition, some patients from Hôpital Bicêtre, Paris, and from CHU Bordeaux, Bordeaux, were recruited.

Altogether, we collected 80 allograft biopsy specimen potentially usable for microarray analyses. Furthermore, from about half of all patients we have concurrently harvested serum samples and aliquots of full blood for the extraction of leukocytes' RNA (PAXGene®, Qiagen).

Among these biopsies, 19 biopsies were taken from nephrectomy specimen of patients suffering from solid renal cancer using an otherwise identical procedure. Healthy looking renal cortex was obtained at maximal distance from circumscribed malignancies. These samples served as control kidney (nephrectomy). Transplant biopsies were classified based on the Banff 97 classification of renal allograft pathology.

Altogether, we had the following three major patient groups: (1) Control patients without a renal transplant (nephrectomy specimen), (2) control patients with a renal transplant (normal tissue, non-immunological lesions, or acute rejection), and (3) patients with chronic allograft nephropathy (CAN; CAN alone, and acute rejection on CAN).

Optimal control samples would consist of perioperative biopsies obtained at day 0 from entirely normal kidneys, preferably from living donation. However, due to restricted availability, we were only able to include two such samples.

RNA was extracted from all biopsy samples.

From 1 mg renal tissue protected in RNAlater (Qiagen), 50 ng to 2.5 mg of total RNA was yielded (RNeasy[®] Micro and Mini Kits; Qiagen). Subsequently, we used an Agilent 2100 Bioanalyzer to ensure high quality starting material for microarrays; exact quantification of RNA was determined with a NanoDrop ND-1000 spectrophotometer (from 1 ml of total RNA). All biopsies resulted in high quality RNA potentially usable for future microarray analyses.

Four different protocols for the processing of the samples were evaluated in a series of preliminary experiments that utilised extracted total RNA from our biopsies. The protocols included the Affymetrix[®] standard protocol, the Nugen Ovation Kit protocol, and Affymetrix[®] amplification protocols with 100 ng and 50 ng of RNA, respectively. As a result, the Affymetrix[®] amplification protocols demonstrated nearly identical correlation coefficients of 0.947 and 0.945, as compared to the standard protocol. In contrast, the Nugen amplification protocol introduced significant changes, reflected by a correlation coefficient of 0.804. Thus, the Affymetrix[®] 50 ng protocol (Affymetrix SST[®]) was selected for the processing of all 80 samples that were available.

During the first half of 2005, all Affymetrix GeneChip[®] HG-U133 Plus 2 Array (microarray) hybridisations were performed at the Novartis Genomics Factory in Basel, Switzerland. These microarrays comprise 54 000 probe sets for 38 500 genes with 47 000 transcripts.

All raw microarray data have already been generated and are currently being analysed in the Department of Biostatistics from Novartis. Thereby, MAS5 normalised gene expression values were subjected to statistical analyses following a three level procedure: statistical filtering (ANOVA), gene set enrichment analysis (GSEA), and use of the nearest shrunken centroid method [16]. Based on our Research Hypothesis, we intend to focus

mainly on metzincins, targets of metzincins and factors involved in their regulation.

Gene expression profiling of chronic allograft nephropathy [22]

First, we investigated whether chronic allograft nephropathy could be distinguished from normal control samples based on the gene expression profile taking into account all genes analysed on the Affymetrix GeneChip[®] HG-U133 Plus 2 Array. These microarray data lead to the identification of a set of 937 genes that are differentially expressed among healthy controls and diagnostic biopsies with chronic allograft nephropathy grade I, II, III (figures 1 and 2), as reported in abstract form [22].

Subsequently, we have started to analyse more closely gene expression profiles of our genes of interest, as stated above. The first series of preliminary results showed that several metzincins, metzincin substrates and related genes were indeed up-regulated in chronic allograft nephropathy. Importantly, a correlation between the degree of up-regulation and the severity of chronic allograft nephropathy existed. In addition, such gene expression profiling may allow the separation of normal tissue from biopsy samples with chronic allograft nephropathy.

Currently, microarray analyses of the concurrently harvested blood samples are in progress. These studies will describe the gene expression profiles of leukocytes for comparison with the tissue samples.

In conclusion, these first results showed that metzincins, especially MMP/TIMP, may be potential markers and therapeutic targets in the case of chronic allograft nephropathy. Altogether, our investigations may help in the search for an early diagnosis and therapy of chronically rejected transplants prior to overt clinical damage.

Figure 1

Dynamic gene-signatures of chronic allograft nephropathy. These signatures are represented by normalised values for gene expression intensity. ANOVA identified a total of 937 genes to be differentially expressed among controls and diagnostic biopsies with CAN grade I, II, III (FDR = 0.05 and FC ≥ 2). Each line in the graph represents one of these genes; with respect to controls, blue lines represent down-regulated genes and red lines depict up-regulated genes in CAN. In this respect, the extent of this down-regulation and up-regulation correlates with the severity of CAN.

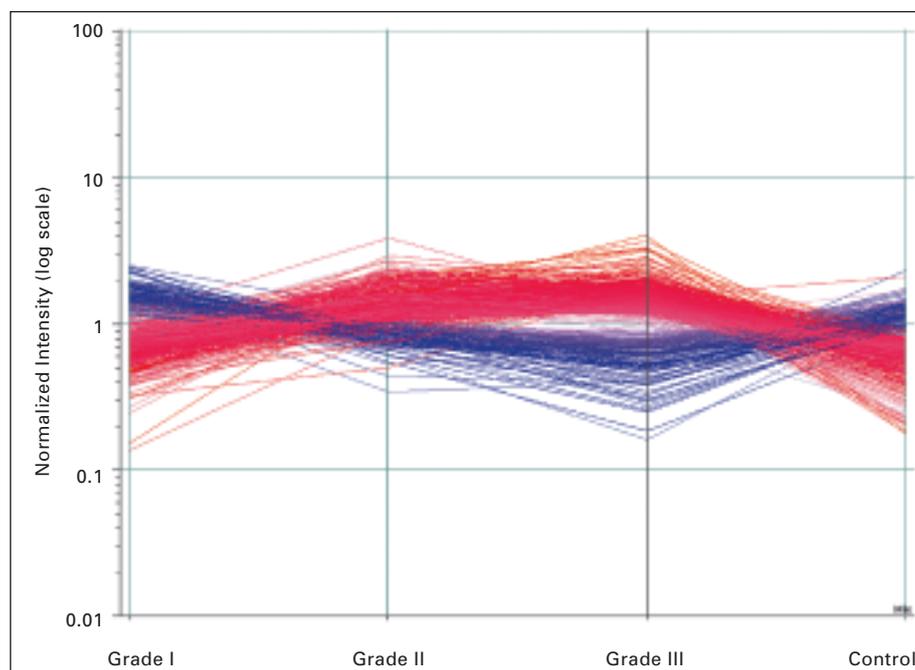
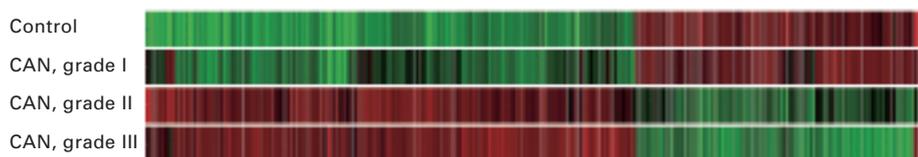


Figure 2

Heat map pattern. Heat map of expression values of 937 genes found to distinguish control biopsies and biopsies with different grades of chronic allograft nephropathy. Each of the four rows depicts these genes. Numerical gene expression values are assigned a colour, where red corresponds to high expression and green corresponds to low expression of that particular gene. Degree of colour saturation reflects the magnitude of the log expression signal. There are clearly visible differences regarding the gene profiling between control, CAN I, CAN II, and CAN III samples. ANOVA, FDR = 0.05 and Fold change ≥ 2 .



Discussion and conclusion

The incidence of end-stage renal disease (ESRD) is growing by an annual rate of 6% to 8% with dialysis remaining the only option for the majority of these patients [23]. Thus, there is a rising demand for refined diagnostic tools and more efficient treatment forms for patients with chronic renal failure, including transplant recipients.

The success of kidney transplantation leading to one-year graft survival rates in the order of 90% correlates with the development of powerful immunosuppressive agents to prevent and to treat acute rejection episodes [24]. However, chronic allograft nephropathy and death with a functioning transplant represent the leading causes of late graft loss of around 3–5% per year [24]. Thus, new strategies to improve long-term graft survival have become the priority in renal transplantation.

There still exists no satisfactory therapy for chronic allograft nephropathy. This disorder mainly includes chronic rejection but also other non-immunologic damage to the transplant. Furthermore, diagnosis is based on observer-dependent interpretation of histological alterations, and patient prognosis often remains ill defined.

Quantitative and qualitative alterations of extracellular matrix proteins and increased proliferation of various cell types are characteristic features of acute and chronic rejection processes. These features are regulated to an important extent by metzincins [1–6]. Members of the metzincin family were found to be good diagnostic and predictive factors in two recent unbiased investigations of tubulo-interstitial nephritis and acute renal allograft rejection [21, 25].

Our own studies support an active role of metzincins (mainly MMP) in chronic renal allograft rejection, including chronic allograft nephropathy in humans. Chronic renal allograft rejection and acute heart transplant rejection, showed in our two rat models a surprisingly good concordance with respect to increased MMP/TIMP regulation; the most significant exceptions were MMP-9 and MMP-11.

However, key immunosuppressive agents like cyclosporine A do not appear to be critical regulators of these enzymes. Thus, increased MMP activities may need to be controlled by separate agents, such as synthetic MMP inhibitors [14, 26].

MMP may act as pro-inflammatory mediators in allograft rejection in several ways: direct tissue injury, augmentation of cell proliferation and/or migration and facilitation of tissue invasion by ex-

trinsic cells. As just recently shown by others, rats in the Lew to F344 chronic rejection model were exposed to an inhibitor of MMP-2,-3 and -9 (BAY 12-9566) during 10 days after transplantation (early treatment) and from week 12 to 20 following transplantation (late treatment) [26]. The early MMP inhibitor application resulted in a significantly reduced proteinuria associated with a lower grade of chronic allograft nephropathy, findings not observed after late MMP inhibition.

The functional role of MMP in human transplantation medicine remains ill defined. To the best of our knowledge, only descriptive studies exist and studies defining an exact biological function of MMP in solid organ transplantation are lacking. For instance, increased circulating levels of MMP-1 were found in human subjects with acute kidney allograft rejection, whereas concentrations of MMP-2 and MMP-3 were augmented in patients with chronic transplant nephropathy [27].

Results of our studies help to better adjust and tailor the medical treatment after kidney transplantation to improve the survival of allografts, especially those with signs of chronic allograft nephropathy. In addition, diagnosis and prognosis may be better refined.

Furthermore, the definition of metzincins and related factors as diagnostic, prognostic and potential therapeutic factors may have consequences beyond chronic allograft nephropathy for kidney diseases in native kidneys or even for inflammatory states in other organs than the kidney.

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Correspondence:

Professor Hans-Peter Marti, MD
Division of Nephrology and Hypertension
Inselspital Berne
University of Berne
CH-3010 Berne
Switzerland
E-Mail: Hans-Peter.Marti@insel.ch

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