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## **Aberrant Tubulointerstitial Immunoexpression of Matrix Metalloproteinases MMP-2, MMP-9 and Tissue Inhibitor of Matrix Proteinase-2 (TIMP-2) in Acute Cellular Rejection of Human Renal Allograft**

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**Acute cellular rejection (ACR) may initiate chronic allograft dysfunction with alterations in the extracellular matrix compartment (ECM). Turnover of ECM proteins is regulated by matrix metalloproteinases (MMPs). The aim of the present study was to determine the immunoexpression of MMP-2, MMP-9 and TIMP-2 in ACR, and to examine the relationship between expression of MMPs and monocytes/macrophages, transforming growth factor  $\beta$ -1 (TGF $\beta$ -1), and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). Immunoperoxidase study with antibodies against MMP-2, MMP-9, TIMP-2, CD68, TGF $\beta$ -1 and  $\alpha$ -SMA was carried out on 24 renal allograft biopsy specimens from patients with ACR and 11 allograft biopsy specimens from patients with no signs of rejection. Our study revealed increased immunoexpression of MMP-2, MMP-9 and TIM-2 in ACR as compared with controls, and significant positive correlations between immunostaining of MMP-2 and TGF- $\beta$ -1, as well as between MMP-2 and  $\alpha$ -SMA. Increased immunoexpression of MMP-9 was positively correlated with  $\alpha$ -SMA, and the number of interstitial CD68+cells. In conclusion our study supports a role of gelatinases in tissue damage in human renal acute cellular allograft rejection and provides some interesting insights into early renal remodeling which may lead to chronic allograft dysfunction.**

### **Introduction**

Acute T-cell-mediated rejection (ACR) is the common form of rejection, mediated by T cells, that develops classically 1 to 6 weeks after transplantation, but may erupt at

any time, even after many years. The hallmark of ACR is a pleomorphic interstitial infiltrate of activated lymphocytes and monocytes, accompanied by interstitial edema and tubular injury. Although acute rejection has had a decreasing incidence over the last decade, it is still a relevant problem in kidney transplantation [23]. It is thought that acute rejection is the major immunologic risk factor for developing chronic renal allograft dysfunction. There is an association between the number and/or severity of acute rejection episodes and decreased probability of long-term graft survival [16]. The principal histopathologic lesion seen in chronic allograft dysfunction is tubular loss with concurrent interstitial fibrosis that leads to renal failure [18]. In acute cellular allograft rejection tissue destruction is associated with qualitative and quantitative alterations in the extracellular matrix compartment (ECM). Under normal conditions, the amount of each protein of the ECM is equilibrium between synthesis and degradation. Matrix metalloproteinases (MMPs) are the most important proteolytic enzymes involved in remodelling of ECM, and are crucial for tissue development and homeostasis. The major natural inhibitors of MMPs are tissue inhibitors of matrix metalloproteinases (TIMPs) which complex with MMPs and are involved in regulating the activity and activation of individual MMPs. MMPs are classified into six groups based on substrate and sequence homology. Gelatinases: MMP-2 (gelatinase A) and MMP-9 (gelatinase B) cleave the denatured collagens (gelatins) and laminin, as well as some chemokines [8]. MMP-2 can also activate MMP-1 and MMP-9 by cleaving their prodomains [30]. Recent study also demonstrated that gelatinases are involved in the process of epithelial to mesenchymal transformation (EMT) in renal tissue [9, 10].

The hallmarks of fibroblast activation, as well as tubular EMT, are de novo expression of  $\alpha$ -smooth muscle actin and overproduction of the interstitial matrix components [21]. Transforming growth factor beta (TGF- $\beta$ ) has been recognized as a key mediator of renal fibrogenesis [14]. In vitro, TGF- $\beta$  as a sole factor can stimulate interstitial fibroblasts, and tubular epithelial cells to undergo myofibroblastic activation or transition [21].

Studies over the past decades has focused on the role of MMPs and TIMPs in chronic lesions of renal allograft [5, 17, 22], but little work at this time evaluate the immunorexpression of MMPs and TIMPs in human acute cellular rejection of renal allograft. In view of the above the aim of the present study was to determine the immunorexpression of MMP-2, MMP-9 and TIMP-2 in human acute cellular renal transplant rejection (ACR). Another purpose of this study was to examine the relationship between expression of MMPs and monocytes/macrophages, transforming growth factor  $\beta$ -1 (TGF $\beta$ -1), and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA).

## Material and Methods

### *Patients*

Twenty-four renal allograft specimens from patients with ACR were examined by percutaneous renal biopsy. All biopsies had been performed solely for diagnostic purposes. All of our patients were adults: the mean age was  $46.1 \pm 11.1$  and the male to female ratio was 14:10. The specimens were taken from 7 days to 4 months after engraftment (mean 35.5 days). Morphological diagnosis of ACR was established independently by two experienced nephropathologists according to Banff 97 criteria [27], and based on light microscopy and immunofluorescence. The standard triple immunosuppression protocol was used in all patients (cyclosporine subsequently adjusted on the basis of trough cyclosporine levels, azathioprine and prednisolone). 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  $41.9 \pm 9.3$ ).

### *Immunohistochemistry*

Paraffin sections were mounted onto superfrost slides, deparaffinized, then treated in a microwave oven in a solution of citrate buffer, pH 6.0 for 20 min and transferred to distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in distilled water for 5 min, and then sections were rinsed with Tris-buffered saline (TBS, DakoCytomation, 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), mouse-anti-human tissue inhibitor of matrix metalloproteinase-2 (clone 46E5, Novocastra Lab, dilution 1:50), polyclonal goat-anti-human TGF- $\beta$ -1 antibody (Santa Cruz Lab, dilution 1:200),  $\alpha$ -SMA (clone P1b5, DakoCytomation, Denmark, dilution 1:50), and monoclonal mouse anti-human CD68 antibody (DakoCytomation, Denmark, dilution 1:100). Afterwards LSAB+/HRP Universal kit (DakoCytomation, 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 (DakoCytomation, Denmark), per ml Tris-HCl buffer, pH 7.6, containing 0.02% hydrogen peroxide, for 10 min. After washing, the sections were counter-stained with hematoxylin and coverslipped. For each antibody and for each sample a negative control were 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, TIMP-2 and TGF- $\beta$ -1 in tubulointerstitium were recorded semiquantitatively by two independent observers in 7-10 adjacent high power fields and graded from 0 (staining not detectable), 1 (minimal immunostaining in some cells), 2 (weak immunostaining intensity in all cells) and 3 (strong staining in all cells). The mean grade was calculated by averaging grades assigned by the two authors and approximating the arithmetical mean to the nearest unity.

### *Morphometry*

Histological morphometry was performed by means of image analysis system consisting of a PC computer equipped with a Pentagram graphical tablet, Indeo Fast card (frame grabber, true-color, real-time), produced by Indeo (Taiwan), and color TV camera Panasonic (Japan) linked to a Carl Zeiss microscope (Germany). This system was programmed (MultiScan 8.08 software, produced by Computer Scanning Systems, Poland) to calculate the number of objects (semiautomatic function) and the surface area of a structure using stereological net (with regulated number of points). The coloured microscopic images were saved serially in the memory of a computer, and then quantitative examinations had been carried out. Interstitial myofibroblasts were identified by their morphology and positive staining with anti- $\alpha$ -SMA. The expression of  $\alpha$ -SMA was measured using point counting method which is an adaptation of the principles of Weibel [31]. The point spacing being  $16 \mu\text{m}$ . Total number of the points of a net was 169, and total area was  $36864 \text{ sq.}\mu\text{m}$ . Under the net described above

8-10 randomly selected adjacent fields of the renal cortex were investigated. Glomeruli and large blood vessels were neglected. As most of the  $\alpha$ -SMA positivity was within cytoplasmic processes, these structures were included in calculation. The percentage of  $\alpha$ -SMA positive staining was an expression of the number of points overlying  $\alpha$ -SMA positive areas as a percentage of the total points counted.

Interstitial monocytes/macrophages were determined by counting CD68+ cells (semiautomatic function) in a sequence of ten consecutive computer images of 400 x high power fields - 0.0047 mm<sup>2</sup> each. The only adjustments of field were made to avoid glomeruli and large vessels. The results were expressed as a mean number of CD68 immunopositive cells per mm<sup>2</sup>.

### Statistical methods

All values were expressed as the mean  $\pm$  SD (standard deviation). The differences between groups were tested using Student 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 ACR group MMP-2, MMP-9 and TIMP-2 was detected in the renal tubular epithelial cells and interstitium (Fig. 1, 2, 3). In controls only slight focal expression of MMP-2, MMP-9 and TIMP-2 in tubular epithelial cells was seen.

The semiquantitative data of the immunoexpression of MMP-2, MMP-9, TIMP-2 and TGF- $\beta$ -1 in tubuli and interstitium, as well as morphometric data of the interstitial CD68+ cells and  $\alpha$ -SMA appear from Table 1. The mean values of the immunoexpression of MMP-2, MMP-9, TIMP-2, TGF- $\beta$ -1,  $\alpha$ -SMA and CD68+ cells were in ACR group significantly increased in comparison with controls. The correlations between the tubulointerstitial immunoexpression of MMP-2, MMP-9 and TGF- $\beta$ -1, CD68+ cells, and immunostaining of  $\alpha$ -SMA are shown in Table 2. In ACR group there were significant positive correlations between immunostaining of MMP-2 and TGF- $\beta$ -1, as well as between MMP-2 and  $\alpha$ -SMA immunoexpression. We did not find significant relationship between MMP-2 and CD68+ cells. Increased immunoexpression of MMP-9 in ACR group was positively correlated with  $\alpha$ -SMA immunostaining and the number of interstitial CD68+ cells. The correlation between immunostaining of MMP-9 and

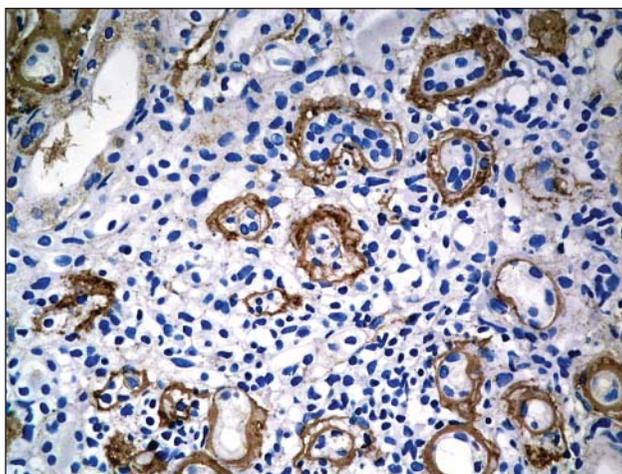


Fig.1. Strong tubular immunoexpression of MMP-2 in kidney biopsy in patient with acute cellular allograft rejection. Immunostaining with antibody against MMP-2. Magnification 400x.

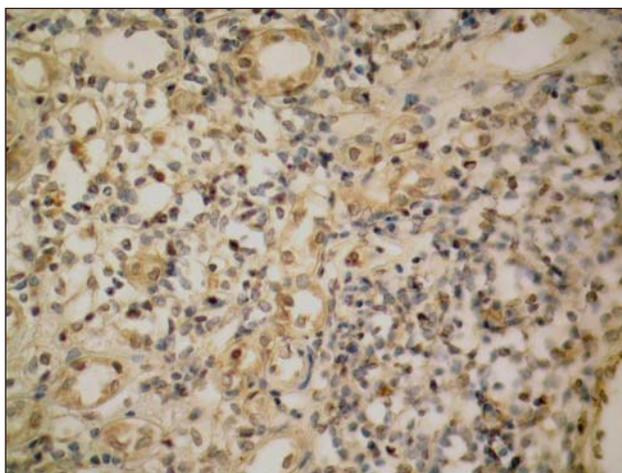


Fig.2. Diffuse immunoexpression of MMP-9 in tubuli and interstitium in kidney biopsy in patient with acute cellular allograft rejection. Immunostaining with antibody against MMP-9. Magnification 400x.

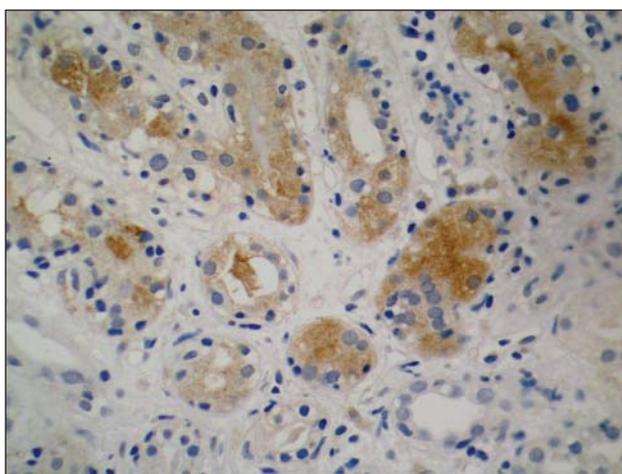


Fig.3. Focal immunoexpression of TIMP-2 in tubuli in renal biopsy in patient with acute cellular allograft rejection. Immunostaining with antibody against TIMP-2. Magnification 400x.

**TABLE 1**

Tubulointerstitial expression of MMP-2, MMP-9, TIMP-2, TGF- $\beta$ -1,  $\alpha$ -SMA and the number of interstitial CD68+ cells in ACR group and controls

Number of cases	MMP-2 (mean score)	MMP-9 (mean score)	TIMP-2 (mean score)	TGF- $\beta$ -1 (mean score)	$\alpha$ -SMA (%)	CD68+ (cells / 1mm <sup>2</sup> )
Controls (n=11)	1.1 $\pm$ 0.46	1.85 $\pm$ 0.78	0.42 $\pm$ 0.12	0.3 $\pm$ 0.2	1.37 $\pm$ 0.58	48.78 $\pm$ 20.67
ACR (n=24)	1.75 $\pm$ 0.87	2.71 $\pm$ 0.96	0.67 $\pm$ 0.37	0.9 $\pm$ 0.7	8.17 $\pm$ 6.27	158.25 $\pm$ 96.21
P value	<0.03	<0.02	<0.04	<0.01	<0.001	<0.001

**TABLE 2**

The correlations between tubulointerstitial expression of MMPs and selected interstitial parameters in ACR group and controls

Correlation between:	ACR (n=24)	Controls (n=11)
tubulointerstitial expression of MMP-2-and TGF- $\beta$	r=0.44, p<0.03	r=0.19, p=0.57 (NS)
tubulointerstitial expression of MMP-2-and $\alpha$ -SMA	r=0.61, p<0.002	r=0.09, p=0.79 (NS)
tubulointerstitial expression of MMP-2-and CD68+ cells	r=0.27, p=0.20 (NS)	r=0.35, p=0.29 (NS)
tubulointerstitial expression of MMP-9-and TGF- $\beta$	r=0.38, p=0.06 (NS)	r=-0.04, p=0.90 (NS)
tubulointerstitial expression of MMP-9-and $\alpha$ -SMA	r=-0.73, p<0.001	r=0.29, p=0.38 (NS)

TGF- $\beta$ -1 cells tended to be positive, however it did not reach statistical significance. In controls all these correlations were not significant.

## Discussion

The regulation of ECM remodeling in the kidney by MMPs has previously been studied by use of models of interstitial and glomerular fibrosis [12, 15]. A number of studies have demonstrated a link between aberrant MMP expression and chronic renal allograft dysfunction, but

conflicting publishing data on MMPs in acute allograft rejection are demonstrated [13, 19]. It is widely accepted that 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 extrinsic cells [5]. Our study revealed increased tubulointerstitial immunoeexpression of MMP-2, MMP-9 and TIMP-2 in ACR group as compared with controls. The two gelatinases MMP-2 and MMP-9 have ability to breakdown components of the basement membrane and collagens, and in vitro both enzymes have a very similar substrate profile [32]. Over a decade ago, Bonventre [7]

postulated a role of proteolysis of tubular cells during acute ischemia-reperfusion injury in the kidney. Basile and al. [3] revealed that MMP-2 and MMP-9 were increased in renal tubules and the interstitium after 1-3 days of reperfusion following ischemia in rats. It is thought that cell adhesion molecules are critical targets of MMPs involved with increased vascular and tubular permeability characteristic of acute kidney injury [8]. The morphology of the rejection process is heterogeneous, which reflects the fact that allograft rejection can be mediated by a variety of immune mechanisms. Surprisingly, recent papers suggest that early changes in the process of acute renal transplant rejection in the first three months after transplantation include interstitial fibrosis [1, 2]. These observations were confirmed by us in our previous study [11]. Matrix-degrading enzymes are historically considered to reduce matrix accumulation, thereby attenuating renal fibrosis after injury. However, recent genetic studies using knock out mice have painted a different and complex picture of the function of these proteins in relation to fibrotic lesions in vivo. MMP-2 is found to be necessary and sufficient to induce tubular EMT in vitro, as well as increased MMP-9 disrupts the integrity of tubular basement membrane, which leads to the promotion of tubular EMT [9, 10, 21]. TGF- $\beta$  is thought to play a crucial role in the induction of EMT within the kidneys [29]. Recent studies suggest that the fibrogenic properties of TGF- $\beta$  are mediated through direct activation of Smad 2/3 [20]. Tubular epithelial injury in acute cellular rejection results in the progressive loss of defined epithelial features and is accompanied by the acquisition of a mesenchymal phenotype, including expression of  $\alpha$ -SMA. Dissolution of the basement membrane by MMP-2 and MMP-9 initiate the cell invasion/migration into the interstitial space. According to the above, our results revealed increased tubulointerstitial immunoexpression of TGF- $\beta$  and  $\alpha$ -SMA in ACR group. Moreover, in ACR group there were significant positive correlations between immunostaining of MMP-2 and TGF- $\beta$ -1, as well as between MMP-2 and  $\alpha$ -SMA immunoexpression. Increased immunoexpression of MMP-9 in ACR group was positively correlated with  $\alpha$ -SMA, and the correlation between immunostaining of MMP-9 and TGF- $\beta$ -1 cells tended to be positive, however it did not reach statistical significance. MMPs, including MMP-2 and MMP-9, have been shown to be involved in tissue invasion of inflammatory cells, such as macrophages, neutrophils, and lymphocytes by several mechanisms. Moreover some of MMP degradation products of matrix proteins function as potent chemoattractants for these cells [26]. Acute cell mediated kidney transplant rejection represents a severe form of inflammation, characterized by tissue invasion of mononuclear leukocytes resulting in cellular and ECM destruction. In our study increased immunoexpression of MMP-9

in ACR group was positive correlated with the number of interstitial CD68+ cells. The activity of MMP-2 is regulated by a natural inhibitor TIMP-2. Experimental studies have shown that TIMP-2 play a bifunctional role in the membrane-type matrix proteinase (MT-MMP) –induced activation of proMMP-2. High doses of TIMP-2 inhibit proMMP-2 activation by MT-MMP in plasma membrane, but TIMP-2 is a requirement for the reaction at low doses [28]. While TIMP-2 inhibits active MT1-MMP and MMP-2 by binding to the active site of the enzymes via its N-terminal inhibitory region, it can also form a non-covalent complex with pro-MMP-2 by binding to the haemopexin-like domain of the zymogen via its C terminal domain [25]. Laplante et al. [19] revealed in experimental study an altered MT1-MMP-dependent processing of proMMP-2 into active MMP-2 due to a diminished TIMP-2 level in acute kidney rejection. Nicholson et al. [24] showed positive correlations between the level of tubulointerstitial collagen III immunostaining and intragraft expression of the genes for TIMP-1 and TIMP-2 in renal transplant fibrosis. In a rat model of acute cardiac allograft rejection mRNA levels of MMP-2, 7, 8, 9, 12, 14 and TIMP-1, 2, 3 were clearly increased [6]. Bernardo et al. [4] investigated the effect of TIMP-2 on MMP-2 activity in the extracellular space in live cells, and showed that both free and inhibited MMP-2 were detected in the medium, and the net MMP-2 activity correlated with the level of TIMP-2 expression. It is worthy of note that the antibody used in our study for immunodetection of MMP-2 does not differentiate between active and inactive forms.

In conclusion our study supports a role of gelatinases in tissue damage in human renal acute cellular allograft rejection and provides some interesting insights into early renal remodeling which may lead to chronic allograft dysfunction.

This work was supported by Medical University in Łódź, grant 502-16-655

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