Monocyte chemoattractant protein-1, but not RANTES plays a crucial role in the interstitial renal injury in IgA nephropathy

OLGA STASIKOWSKA
MARIAN DANILEWICZ
MAŁGORZATA WĄGROWSKA-DANILEWICZ

Medical University of Lodz
Department of Nephropathology
92–216 Lodz, ul. Czechosłowacka 8/10
POLAND

Correspondence:
Olga Stasikowska
Medical University of Lodz
Department of Nephropathology
92–216 Lodz, ul. Czechosłowacka 8/10
POLAND

Abbreviations:
IgAN   – IgA nephropathy
RANTES – Regulated upon Activation Normal
          T-cell Expressed and Secreted
MCP-1   – monocyte chemoattractant protein-1
TGF-β1 – transforming growth factor-β1
α-SMA – α-smooth muscle actin

Key words: MCP-1, RANTES, tubulointerstitial injury, IgA nephropathy

Abstract

Background and Purpose: IgA nephropathy (IgAN) is the most common glomerulonephritis worldwide, and one of the major causes of end-stage renal disease. Approximately one-third patients with IgAN developed chronic renal failure over 30-year follow-up period. Tubulointerstitial infiltrations are found during progression of IgAN. Interstitial infiltrating immune cells could be recruited within interstitial areas by two powerful chemoattractants: monocyte chemoattractant protein-1 (MCP-1) and Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES). Infiltrating macrophages are a significant source of transforming growth factor-β1 (TGF-β1) modulating the interstitial myofibroblasts which express α-smooth muscle actin (α-SMA) activity. The present study was undertaken to evaluate the possible relationships between the immunoexpression of chemokines and their relationship with interstitial renal fibrosis in IgAN.*

Materials and Methods: Paraffin-embedded renal biopsy specimens of 19 patients with IgA nephropathy (diffuse mesangial proliferation, IV subclass of IgAN), and 10 normal kidneys were retrospectively studied by immunohistochemistry, using antibodies against MCP-1, RANTES, CD68, TGF-β1, α-SMA.*

Results: In the renal tissue in IgAN the tubulointerstitial immunoexpression of MCP-1 and RANTES was increased as compared with normal controls. The tubulointerstitial MCP-1 immunoexpression in IgAN was significantly correlated with the number of CD68+cells, immunoexpression of TGF-β1 and α-SMA in tubuli and interstitium, as well as with renal cortical volume, serum creatinine level and proteinuria. Statistical analysis did not revealed significant correlations between RANTES immunoexpression and the studied parameters.*

Conclusions: Based on the findings of this article we suggest that chemokine MCP-1 via TGF-β1 plays an important role in the interstitial renal fibrosis in IgA nephropathy.*

INTRODUCTION

IgA nephropathy (IgAN) is one of the most common forms of primary glomerulonephritis in adults. As observed in most types of glomerulonephritis, interstitial accumulation of monocytes/macrophages and T lymphocytes is common in IgAN *(1)*. Infiltrating and proliferat-

ing cells can contribute to the progression of IgAN towards end-stage renal failure *(2, 3)*. Moreover, severe proteinuria and persistently elevated serum creatinine are associated with a poor prognosis. Selective attraction of different subset of leukocytes to the site of inflammation appears to be mediated to a significant extent by the expression of specific chemokines and chemokine receptors. Human chemokines represent at least 40 different chemotactic proteins that share conserved structural features including four cysteine residues that help define the chemokine subfamilies. These include the C, CC, CXC and CX3C subfamilies. Chemokines act by binding to chemokine receptors on target cells *(4, 5)*. Important members of the CC subfamily are MCP-1 (monocyte chemoattractant protein-1) and RANTES (Regulated upon Activation Normal T-cell Expressed and Secreted). Chemokines and chemokine receptors are involved at multiple stages of inflammatory response in renal disease, including leukocyte adhesion, transmigration and differentiation into the tissue specific phenotype. Monocytes/macrophages and T cells releasing reactive oxygen species, cytokines, growth factors, and autocaids can damage or activate tubular and interstitial cells. Important feature of tubulointerstitial inflammation is the presence and activation of interstitial fibroblasts. Two mechanisms can cause fibroblast multiplication: proliferation of resident interstitial fibroblasts and transformation of tubular epithelial cells into fibroblasts. The transdifferentiated epithelial cells and resident interstitial fibroblasts may express a cytoplasmic marker of fibroblast activation alha-smooth muscle actin (α-SMA) *(2)*. Activated fibroblasts may synthesize extracellular matrix, playing a major role in the pathogenesis of interstitial fibrosis *(6)*.

In this study we analyzed the immunoexpression of chemokines: MCP-1 and RANTES and their relationship to intensity of monocyte/macrophage infiltrate, the immunoexpression of TGF-β1, α-SMA, and the value of interstitial cortical volume, as well as serum creatinine level and proteinuria in patients with IgA nephropathy.

MATERIALS AND METHODS

Patients

Renal tissue biopsies were obtained percutaneously for diagnostic purposes from 19 patients (7 females and 12 males, aged 21–53, mean age=25) with IgAN. Laboratory data including urinalysis, 24h protein excretion and serum creatinine level were collected from each patient. At the time of biopsy 4 patients presented nephrotic range proteinuria, in 7 patients proteinuria was higher than 2g/24h, and in 8 cases proteinuria was up to 2g/24h. Hematuria was noted in all IgA nephropathy patients. Renal function impairment was observed in 5 patients. In all cases diagnosis of glomerulonephritis was based on characteristic findings by light microscopy (sections stained with Hematoxylin and Eosin, Masson-Trichrome, Jones' silver impregnation and periodic acid--Schiff followed by Alcian Blue), immunofluorescence and electron microscopy using standard protocols. In all

renaI biopsy samples in patients with IgAN diffuse mesangial proliferation was observed (IV subclass of IgAN, according to Haas system) *(7)*. As a control 10 biopsy specimens of the kidneys removed because of trauma were used. None of the persons from control group was known to have had previous or actual renal disease. Before the quantitative examination was carried out, all control specimens were examined by an experienced nephropathologist and found to be a normal renal tissue.

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: goat anti-human MCP-1 antibody (R&D Systems, UK&Europe), dilution 15 μg/mL; polyclonal goat anti--human RANTES antibody (R&D Systems, UK&Europe), dilution 10 μg/mL; monoclonal mouse anti-human CD68 antibody (DakoCytomation, Denmark), dilution 1:200; polyclonal rabbit anti-human TGF-β1 antibody (Santa Cruz Biotechnology, inc.), dilution 1:200, and monoclonal mouse anti-human α-SMA (DakoCytomation, Denmark), dilution 1:200. Afterwards LSAB+/HRP Universal kit (DakoCytomation, Denmark) and Cell and Tissue Staining Kit (R&D Systems, UK&Europe) were used prepared according to the instructions of the manufacturer. Visualisation was performed by incubating the sections in a solution of 3,3'-diaminobenzidine (DakoCytomation, Denmark). After washing, the sections were counter-stained with hematoxylin and coverslipped. For each antibody and for each sample a positive control and negative control were processed. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

Staining intensities of MCP-1, RANTES and TGF-β1 were recorded by two independent observers and graded from 0 (negative), 1 (weakly positive), 2 (moderately positive) and 3 (strongly positive). The mean grade was calculated by averaging grades assigned by the two authors and approximating the arithmetical mean to the nearest unity. Tubular and interstitial staining was scored in 10 consecutive high power fields, avoiding glomeruli.

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 Panasonic camera (Japan) linked to a Carl Zeiss microscope (Germany). This system was programmed (program MultiScan 8.08, produced by Computer Scanning Systems, Poland) to calculate the number of objects (semiautomatic function) and the surface area of a structure using stereological net. The coloured microscopic images were saved serially in

TABLE 1

The tubulointerstitial immunoexpression of MCP-1, RANTES, CD68, TGF-β1, α-SMA, and the values of interstitial cortical volume of renal tissues of patients with IgA nephropathy (IgAN) and of normal controls.

Number of cases	MCP-1	RANTES	CD68	TGF-β1	α-SMA	Interstitial cortical volume
IgAN (n=19)	1.21±0.74	1.17±0.78	68.40±28.12	1.2±0.68	5.84±2.93	10.91±1.59
Normal control (n=10)	0.38±0.26	0.05±0.08	33.99±28.06	0	0.52±0.26	20.37±9.09
P value	P<0.003	P<0.001	P<0.002	P<0.001	P<0.001	P<0.004

Data are expressed as mean values ±SD

computer memory, and then quantitative examinations carried out. Interstitial myofibroblasts were identified by their morphology and positive staining with anti--α-SMA. The immunoexpression of α-SMA was measured as a surface fraction using point counting method which is an adaptation of the principles of Weibel *(8)*. The point spacing was 16 µm. Total numbers of the points of a net was 169, and total area was 36864 sq. µm. 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 α-SMA immunopositivity was within cytoplasmic processes, these structures were included in calculation. The percentage of α-SMA positive staining was an expression of the number of points overlying α-SMA positive areas as a percentage of the total points counted. The same method was used to estimate interstitial volume in sections stained with Masson trichrome. The percentage interstitial volume was an expression of the number of points overlying renal cortical interstitium i.e. a percentage of the total points counted.

Interstitial CD68+ cells were determined by counting (semiautomatic function) in a sequence of ten consecutive computer images of 400 x high power fields – 0.0047 mm^2 each. The results were expressed as a mean number of CD68 immunopositive cells per mm^2.

Statistical analysis

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

RESULTS

The data on immunoexpression of MCP-1, RANTES, CD68, TGF-β1, α-SMA, and value of renal cortical volume are shown in Table 1. The immunoexpression of MCP-1 (Figure 1), and RANTES was observed on interstitial mononuclear inflammatory cells and tubular epithelium. In renal tissues in IgAN the tubulointerstitial immunostaining for MCP-1 and RANTES were increased as compared with normal control (P<0.003, P<0.001,

respectively). None of the control renal biopsy specimens provided evidence of TGF-β1 immunopositivity, whereas the immunoexpression of TGF-β1 was detected in tubular epithelial cells (Figure 2), and interstitium in renal biopsy specimens in patients with IgAN. In the renal biopsy specimens in patients with IgAN the interstitial immunoexpression of α-SMA (Figure 3) was increased as compared with control group (P<0.001). The immunoexpression of CD68 was detected on mononuclear
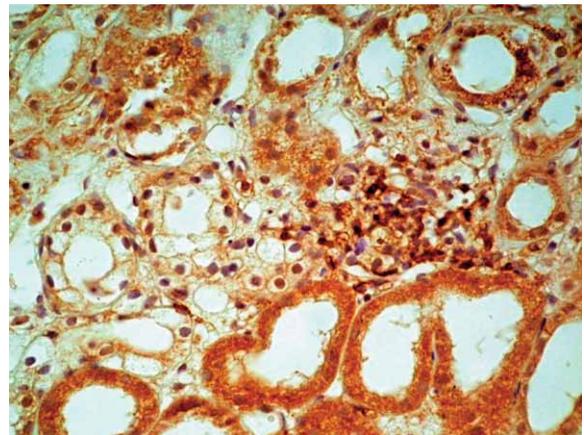

Figure 1. *MCP-1 immunostaining in renal tubuli and interstitial inflammatory cells in biopsy specimen of a patient with IgA nephropathy. Magn. 400x.*
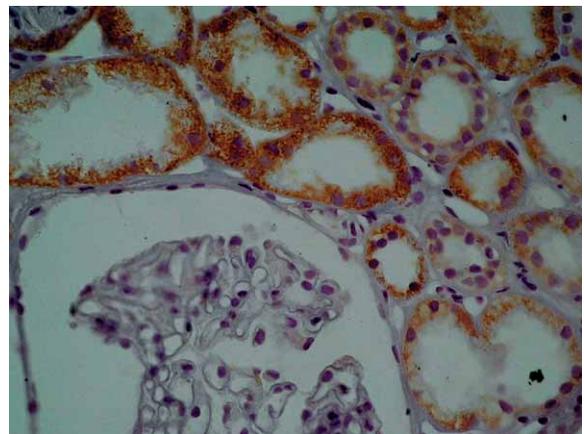

Figure 2. *The immunoexpression of TGF-β1 in the tubular epithelial cells in renal biopsy specimen of a patient with IgA nephropathy. Magn. 400x.*

The correlations between immunoexpression of MCP-1 and CD68+ cells, TGF-β1, α-SMA, the value of interstitial cortical volume, proteinuria and serum creatinine level in patients with IgA nephropathy.

Correlations between:	
MCP-1 and CD68+	r=0,84, P<0.001
MCP-1 and TGF-β1	r=0.63, P<0.004
MCP-1 and α-SMA	r=0.49, P<0.04
MCP-1 and interstitial cortical volume	r=0.55, P<0.02
MCP-1 and proteinuria	r=0.82, P<0.001
MCP-1 and serum creatinine level	r=0.48, P<0.04

TABLE 3

The correlations between immunoexpression of RANTES and CD68+ cells, TGF-β1, α-SMA, the value of interstitial cortical volume, proteinuria and serum creatinine level in patients with IgA nephropathy.

Correlations between:	
RANTES and CD68+ cells	r=0.19, P=0.43, NS
RANTES and TGF-β1	r=−0.18, P=0.43, NS
RANTES and α-SMA	r=0.08, P=0.73, NS
RANTES and interstitial cortical volume	r=0.07, P=0.79, NS
RANTES and proteinuria	r=−0.17, P=0.47, NS
RANTES and serum creatinine level	r=0.17, P=0.47, NS

inflammatory cells infiltrating renal interstitium (Figure 4). The number of interstitial CD68+ cells, and the value of interstitial cortical volume in IgAN patients was increased as compared with control group (P<0.002, and P<0.004, respectively). In patients with IgAN, tubulointerstitial immunostaining of MCP-1 was positively correlated with the number of CD68+ cells (P<0.001), the immunoexpression of TGF-β1 (P<0.004), the immunoexpression of α-SMA (P<0.04), the value of renal cortical volume (P<0.02), proteinuria (P<0.001), and serum creatinine level (P<0.04) (Table 2). Statistical analysis did not reveale significant correlations between RANTES immunoexpression and all observed parameters (Table 3).

DISCUSSION

Our study demonstrated an increase in tubulointerstitial MCP-1 and RANTES immunostaining in renal biopsy specimens in patients with IgAN as compared with normal controls. MCP-1 and RANTES immunoexpression was observed on interstitial mononuclear cells and tubular epithelium. Grandaliano *et al. (9)* observed the same pattern of MCP-1 expression in IgAN, and showed an increased MCP-1 gene and protein expression mainly in cortical tubular epithelial cells, infiltrating mononuclear cells and glomerular parietal cells. Cockwell *at el. (10)* detected RANTES in both glomerular and tubulointerstitial compartment, but RANTES immunoexpression was described only in 11% of the glomeruli. It is though that MCP-1 and RANTES may via the chemokine receptors on the inflammatory infiltrating cells participate in the pathogenesis of glomerular and interstitial lesions in human glomerular diseases *(11–13)*. In the present study immunohistochemical staining confirmed immunoexpression of MCP-1 and RANTES in the tubulointerstitium in the renal biopsy specimens in patients with IgAN. Moreover, immunoexpression of MCP-1 was associated with the presence of infiltrating monocytes/macrophages, and was positively correlated with the degree of tubulointerstitial damage and decrease in renal function. MCP-1- directed chemotaxis of macrophages may
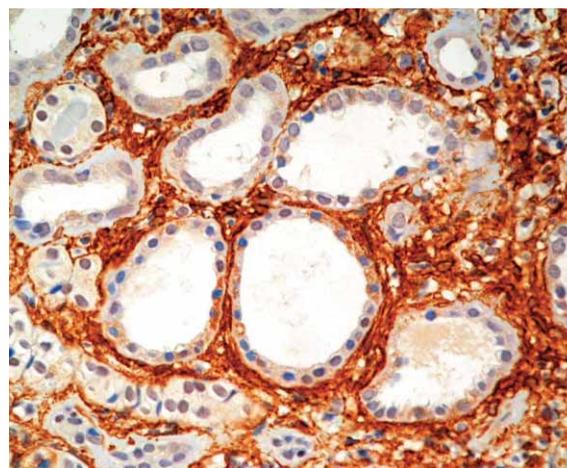

Figure 3. *Strong immunoexpression of α-SMA in the interstitium in renal tissue of a patient with IgA nephropathy. Magn. 400x.*
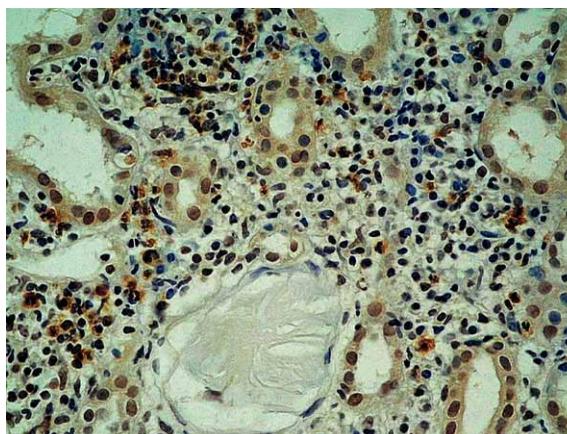

Figure 4. *Numerous CD68 positive cells in the renal interstitium in biopsy specimen of a patient with IgAN. Magn. 400x.*

be of central importance in renal diseases because macrophages probably play a pivotal role in the pathophysiological mechanisms of tubulointerstitial inflammation and fibrosis. Infiltrating activated macrophages and T cells are presumed to be important in tubulointerstitial dysfunction possibly by secreting inflammatory mediators. The macrophages infiltrating renal tissue may be a source of cytokines and growth factor such as TGF-β1, PDGF (platelet-derived growth factor) and IL-1 *(14)*, which plays an essential role in renal damage. TGF-β1 is the main fibrogenic growth factor and it is implicated in the pathogenesis of renal fibrosis in experimental and human nephropathies *(15, 16)*. It is known, that TGF-β1 is a major inducer of transformation of fibroblasts to the α-SMA-positive myofibroblasts that synthesize interstitial extracellular matrix. In our study the tubulointerstitial MCP-1 immunoexpression was significantly correlated with the tubulointerstitial immunoexpression of TGF-β1, α-SMA and interstitial cortical volume. Mezzano *et al.* *(17)* described a strong immunoexpression of MCP-1, RANTES and TGF-β1 which significantly correlates with degree of tubulointerstitial damage and with the presence of interstitial myofibroblasts in patients with membranous nephropathy. Experimental study revealed that MCP-1 mediates collagen deposition in experimental glomerulonephritis by TGF-β1 *(18)*. In addition TGF-β1 may contribute to the secretion of tubular MCP-1 in nephrotic syndrome *(19)*. In our study statistical analysis did not reveal correlations between immunoexpression of RANTES and studied interstitial parameters, as well as proteinuria and serum creatinine levels. To our knowledge, the data concerning the association of RANTES immunoexpression in renal tissue with progression to fibrosis are scanty. Kuroiwa *et al.* *(20)* suggest that RANTES may play a predominant role in migration and activation of T cells rather than macrophages, but T cells infiltrating the interstitium probably activate interstitial macrophages. Previous investigation from our laboratory has demonstrated that RANTES was in lupus nephritis, involved in the pathogenesis of macrophage-induced renal damage *(21)*.

Experimental and human data have documented that progressive proteinuria is associated with tubulointerstitial lesions and renal scarring. The mechanisms by which proteinuria could cause interstitial inflammation and fibrosis are still not fully understood. In the present study the tubulointerstitial MCP-1 immunoexpression was significantly correlated with proteinuria in IgAN patients. It appears that certain urinary proteins (albumin and transferrin) can stimulate proximal tubular cells to synthesis chemokines MCP-1 and RANTES *(22–24)*. Shimizu *et al.* *(25)* postulated that the proteins filtered through glomeruli activate tubular epithelial cells, which secrete vasoactive and inflammatory substances including chemokines, leading to tubulointerstitial renal injury. In addition, specific neutralization of chemokines: MCP-1 and MIP-1a prevented proteinuria and renal dysfunction in experimental crescentic glomerulonephritis models *(25, 26)*.

In conclusion, our results suggest that MCP-1 but not RANTES is involved in the pathogenesis of renal fibrosis, and promotes renal dysfunction in IgAN. This supports a central role of MCP-1, as a unique chemotactic member of the cytokine family, in the progression of inflammatory renal disease and renal fibrosis.

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