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CC Chemokines and Chemokine Receptors in IgA Nephropathy (IgAN) and in non-IgA Mesangial Proliferative Glomerulonephritis (MesProGN). The Immunohistochemical Comparative Study*

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The aim of the study was to compare the immunoexpression of monocyte chemoattractant protein-1 (MCP-1), monocyte inflammatory protein-1α (MIP-1α), Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES) and CC chemokine receptors: CCR1 and CCR5 in renal biopsy specimens in IgA nephropathy (IgAN) and in non-IgA mesangial proliferative glomerulonephritis (MesProGN), and to find any relationships between the immunoexpression of chemokines and chemokine receptors and renal interstitial lesions. Our study revealed increased immunoexpression of tubulointerstitial MCP-1 (P<0.02), RANTES (P<0.03) and interstitial CCR5+ cells (P<0.05) in IgAN as compared with MesProGN. In the renal tissue in patients with IgAN the intensity of tubulointerstitial immunostaining of MCP-1 and the number of CCR1+ cells and CCR5+ cells were significantly correlated with the renal interstitial cortical volume, meanwhile in biopsy specimens from patients with MesProGN only interstitial CCR5+ cells were positively correlated with the interstitial cortical volume. In conclusion, these observations suggest that in the renal tissue in IgAN patients MCP-1, RANTES and CCR5 are up-regulated as compared with renal biopsy specimens from patients with MesProGN. Moreover, MCP-1 as well CCR5 and CCR1 positive cells may play a role in the interstitial processes leading to fibrosis in IgAN, whereas in MesProGN CCR5-positive cells may participate in interstitial lesions in renal tissue.

Introduction

The infiltration of leukocytes into the glomerulus is a mainstay of inflammatory glomerular damage in proliferative glomerulonephritis. Attraction of circulating leukocytes in inflammatory kidney diseases has been proposed to be caused by chemokines. Chemotactic cytokines or chemokines are a group of small cytokines with a molecular weight that ranges from 8 to 10 kDa. Chemokines are characterized by a series of shared structural determinants including conserved cystein residues that form disulfide bonds in the chemokine tertiary structure. According to the positioning of the first two closely paired and highly conserved cysteins of the amino acid sequence the chemokine superfamily can be divided into four groups [1, 8, 16, 19]. In the CC or β chemokines the first two conserved cystein residues are adjacent one another. To the CC-class chemokines belong the monocyte-chemoattractant proteins (MCP-1, 2, 3), macrophage inflammatory proteins (MIP-1α and 1β), RANTES (Regulated upon Activation Normal T-cell Expressed and Secreted). Chemokines act by binding to chemokine receptors (CCR) on target cells. Chemokine receptors are classified according to their ligands into five families. The nomenclature system is rooted by the chemokine subclass specificity of the receptor. Human CC chemokine receptor names consist of the root CCR, followed by a number [9, 10]. These chemokine receptors usually bind more than one chemokine of the same subgroup. The variable expression of the receptors by distinct leukocyte subsets is an important component of the specificity of chemokine action [9, 10, 19]. Cytokines, mesangial cell growth, extracellular matrix are the main factors of the glomerular pathology, which is characteristic for several forms of glomerulonephritis including IgA nephropathy (IgAN) and non-IgA mesangial proliferative glomerulonephritis (MesProGN). In the first stage of the disease only the inflammatory mediators are produced locally by resident cells with autocrine activity and no infiltrating cells are noticed within the kidney. The

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appearance of monocytes/macrophages and lymphocytes in glomeruli and tubulointerstitium is a critical step in the pathogenesis of those diseases.

To date, the classic perception of CC-chemokines was that their main property is the attraction of monocytes/macrophages, but there is an increasing body of evidence that CC chemokines play a major role in the pathogenesis of renal failure progression [16, 19] The objectives of the present study were to compare the immunoexpression of CC chemokines and chemokine receptors in IgAN and in MesProGN, and moreover, to find any relationships between immunoexpression of chemokines and chemokine receptors and the renal interstitial lesions.

Material and Methods

Patients

Kidney tissue biopsies were obtained for diagnostic purposes percutaneously from 19 patients (12 males and 7 females, aged 21–53, mean age=25) with IgAN and from 23 patients (8 males and 15 females, aged 19–56, mean age=31) with MesProGN. Laboratory data including urinalysis, 24 h protein excretion and serum creatinine level were collected from each patient. At the time of biopsy 4 IgAN patients and 8 MesProGN patients presented nephrotic range proteinuria. In 7 IgAN patients proteinuria was more than 2 g/24 h, and in 8 cases proteinuria was up to 2 g/24 h. In 12 patients with MesProGN proteinuria was more than 2 g/24h, and in 3 cases proteinuria was up to 2 g/24 h. Hematuria was noted in all IgAN patients and in 14 patients with MesProGN. Renal function impairment was noted in 5 patients with IgAN and in 4 patients with MesProGN.

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. Classification of the histopathological lesions refers to that of the World Health Organization [2]. In all biopsy samples in patients with MesProGN and IgAN diffuse mesangial proliferation was seen.

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 in Tris-buffered saline (TBS, DakoCytomation) and incubated with polyclonal goat anti-human antibodies (R&D Systems): MCP-1 (dilution: 15 μg/ml:), MIP-1α (dilution: 15 mg/ml), RANTES (dilution: 5 μg/ml), and monoclonal mouse anti-human antibodies (R&D Systems): CCR1 (dilution: 8 μg/ml), CCR5 (dilution: 5 μg/ml) for 30 min. Afterwards, R&D Systems Cell and Tissue Staining Kits/HRP-DAB prepared according to the instructions of the manufacturer was used. Visualization was performed by incubating the sections in a solution of 0.5 mg 3,3'-diaminobenzidine 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 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, MIP-1α and RANTES were recorded by two independent observers and graded semiquantitatively as: 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. Glomerular staining was scored in all glomeruli within renal biopsy specimens. 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 IBM-compatible 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 (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 (with regulated number of points). The colored microscopic images were saved serially in the memory of the computer, and then quantitative examinations had been carried out. Interstitial volume in the sections stained with Masson trichrome was measured using point counting method, which is an adaptation of the principles of Weibel [22]. The point spacing was 16 μm. The number 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. The percent interstitial volume was an expression of the number of points overlying renal cortical interstitium as a percentage of the total points counted.

Quantification of interstitial CCR1+ cells and CCR5+ cells were determined by counting these cells (semiautomatic function) in a sequence of ten consecutive computer images of 400x high power fields – 0.0047 mm^2 each. The results were expressed as a mean number of CCR1 and CCR5 – 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'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 Sperman's method. Results were considered statistically significant if $P<0.05$.

Results

In the renal tissue from patients with IgAN as well as MesProGN no glomerular immunostaining for MIP-1α, RANTES, CCR1 and CCR5 was found. The data of the glomerular and tubulointerstitial expression of MCP-1 and tubulointerstitial immunoexpression of MIP-1α and RANTES appear from Table 1. A very faint staining for MCP-1 in glomeruli in both studied groups was observed (0.23±0.19 in IgAN, and 0.17±0.17 in MesProGN). The glomerular immunostaining of MCP-1 was confined to mesangium. There was no significant difference in glomerular MCP-1 immunostaining in IgAN and MesProGN groups (P=0.29). The immunoexpression of MCP-1, MIP-1α and RANTES was seen on interstitial mononuclear inflammatory cells and tubular epithelium in renal biopsies in both studied groups (Figs. 1–3). In the renal tissue in IgAN the tubulointerstitial immunoexpression of MCP-1 and RANTES was increased as compared with MesProGN ((P<0.02 and P<0.03, respectively) There was no significant difference in tubulointerstitial staining of MIP-1α between IgAN and MesProGN groups (P=0.65).

The morphometric data on the interstitial immunoexpression of CCR1 and CCR5, as well as the values of the interstitial cortical volume appear from Table 2. The immunoexpression of CCR1 and CCR5 were detected on mononuclear inflammatory cells infiltrating renal interstitium (Fig. 4). The number of CCR1-positive cells was similar in renal biopsy specimens in IgAN and MesProGN patients (0.81±0.78, and 0.74±0.68, respectively, P=0.75, NS). As is shown in Table 2, in IgAN group the mean value of interstitial CCR5-positive cells was increased in

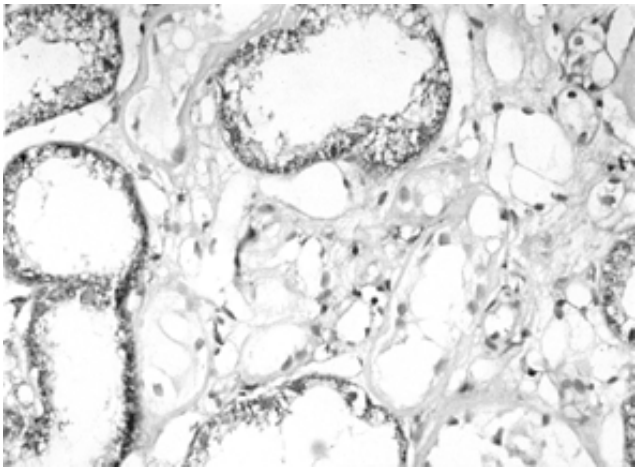

Fig. 1. Focal immunoexpression of RANTES in tubular epithelium in the renal biopsy specimen in patient with IgAN. Magn. 400×.

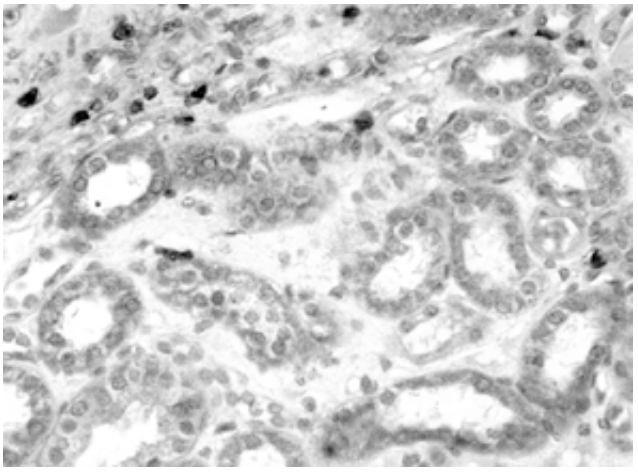

Fig. 2. The immunoexpression of MCP-1 on interstitial inflammatory cells and on tubular epithelial cells in the renal biopsy in MesProGN. Magn.400×.

TABLE 1

The immunoexpression of MCP-1, MIP-1α, and RANTES in renal tissue in IgAN and MesProGN patients

Number of cases	MCP-1 (in glomeruli)	MCP-1 (in tubulo-interstitium)	MIP-1α (in tubulo-interstitium)	RANTES (in tubulo-interstitium)
IgAN (n=19)	0.23±0.19	1.21±0.88	0.41±0.44	1.17±0.92
MesProGN (n=23)	0.17±0.17	0.61±0.61	0.49±0.63	0.64±0.55
P value	P=0.29 (NS)	P<0.02	P=0.65 (NS)	P<0.03

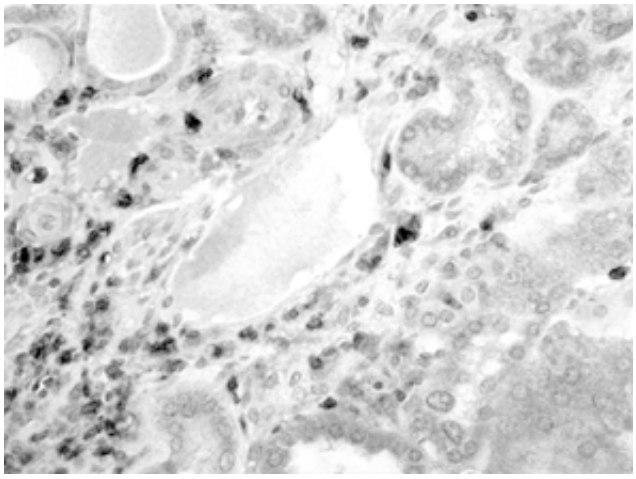

Fig. 3. The immunoexpression of MCP-1 on numerous interstitial inflammatory cells and in tubular epithelium in the renal tissue in IgAN patient. Magn. 400×.

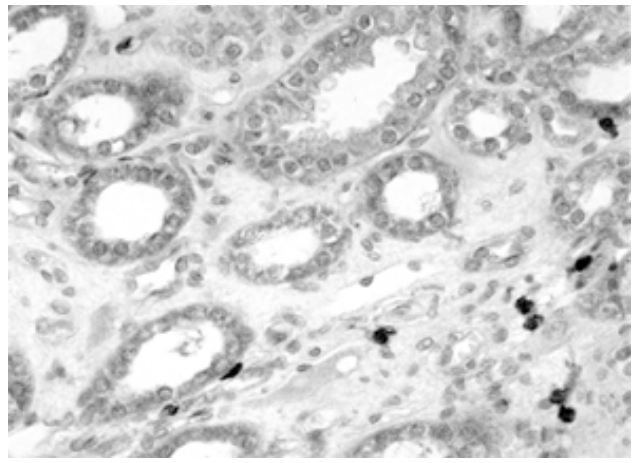

Fig. 4. CCR5-immunopositive cells in the interstitium in renal biopsy specimen in patient with IgAN. Magn. 400×.

TABLE 2

The interstitial CCR1-positive and CCR5-positive cells, and the values of the cortical interstitial volume in patients with IgAN and MesProGN

Number of cases	CCR1+ cells (in the interstitium)	CCR5+ cells (in the interstitium)	Interstitial cortical volume
IgAN (n=19)	0.81±0.78	1.04±0.79	20.38±5.07
MesProGN (n=23)	0.74±0.68	0.60±0.58	17.0±5.05
P value	P=0.75 (NS)	P<0.05	P<0.05

TABLE 3

The correlations between tubulointerstitial immunoexpression of MCP-1, MIP-1α, RANTES, CCR1, CCR5 and the interstitial cortical volume in renal tissue in patients with IgAN and MesProGN

Correlation between:	IgAN	MesProGN
MCP-1 and interstitial cortical volume	r=0.55, P<0.02	r=0.36, P=0.09 (NS)
MIP-1α and interstitial cortical volume	r=0.34, P=0.14 (NS)	r=0.04, P=0.83 (NS)
RANTES and interstitial cortical volume	r=0.07, P=0.76 (NS)	r=-0.2, P=0.32 (NS)
CCR1+ cells and interstitial cortical volume	r=0.51, P<0.03	r=0.08, P=0.08 (NS)
CCR5+ cells and interstitial cortical volume	r=0.52, P<0.03	r=0.59, P<0.003

comparison with the renal tissue from MesProGN patients (P<0.05).

In patients with IgAN the intensity of tubulointerstitial immunostaining of MCP-1 and the number of CCR1+ cells and CCR5+ cells were significantly correlated with the renal interstitial cortical volume (P<0.02, P<0.03 and P<0.03, respectively), whereas in biopsy specimens from patients with MesProGN only interstitial CCR5+ cells were positively correlated with the interstitial cortical volume (P<0.003) (Table 3).

Discussion

Our study revealed increase in the tubulointerstitial immunoexpression of MCP-1, RANTES and the number of interstitial CCR5-positive cells in the renal tissue in IgAN patients as compared with MesProGN. There was no significant difference in the tubulointerstitial staining of MIP-1α between IgAN and MesProGN group. Our study did not reveal immunopositivity for MIP-1α and RANTES within glomeruli in the renal biopsy specimens in IgAN and

MesProGN patients. Moreover, there was no difference in glomerular immunostaining of MCP-1 between both studied groups, and the immunoexpression of this chemokine in glomeruli was scant. This observation is in concordance with the results of Prodjosudjadi et al. [11] who have reported that MCP-1 was expressed primarily at the tubulointerstitial level in different glomerular diseases. Grandaliano et al. [5] observed the same pattern of MCP-1 expression in IgAN. Numerous data suggest that MCP-1, MIP-1α and RANTES *via* the chemokine receptors participate in the pathogenesis of glomerular and interstitial lesion in human glomerular diseases by recruiting and activating monocytes/macrophages [4]. Grandaliano et al. [5] showed an increased MCP-1 gene and protein expression in IgAN, mainly in cortical tubular epithelial cells, infiltrating mononuclear cells, as well as glomerular parietal cells. This expression positively correlated with the presence and the extent of monocyte infiltration and with the severity of tubulointerstitial lesions. Prodjosudjadi et al. [11] analyzed MCP-1 protein expression at glomerular and interstitial sites, but found no correlation between glomerular MCP-1 expression and intraglomerular macrophage infiltration. It is obvious that immunoexpression of MCP-1 is found in glomeruli from patients with glomerulopathies with monocyte infiltrate, but it must be taken into consideration that residual glomerular cells may be a source of chemokines. Rovin et al. [13] demonstrated *in vivo* by immunohistochemistry the expression of MCP-1 in experimental and human glomerulonephritides, but were unable to identify the cellular source of MCP-1. Several stimuli, such as cytokines (that is interleukin-1 and tumor necrosis factor-α) can induce MCP-1 production by mesangial cells [14, 15]. Experimental studies have demonstrated that certain urinary proteins can stimulate proximal tubular cells to synthesize chemokines: MCP-1, RANTES and fractalkine that recruit monocytes and T-cells and interleukin-8 that attracts neutrophils [21, 23]. *De novo* tubular production of MCP-1 has been observed in several proteinuric renal diseases including overload proteinuria [3]. According to the hypothesis proposed by Remuzzi [12] proteinuria itself may induce tubular cell activation and generation of chemokines by proximal tubular cells. In these chronic proteinuric nephropathies in animals and humans the progression to renal parenchymal damage and end-stage renal disease appears to be relatively independent of the initial insults. In our study nephritic range proteinuria was noted in 4 patients with IgAN and in 8 patients with MesProGN. Recent data point that locally produced MCP-1 seems to participate in cell infiltration and interstitial fibrosis from the acute to the chronic phase of renal diseases. Experimental study revealed that MCP-1 has a fibrogenic effect through the stimulation of transforming growth factor-β (TGF-β) [17]. It is suggested that both fibroblasts themselves and fibroblasts co-cultured with immune-inflammatory cells have the ability to participate in the maintenance of an inflammatory response *via* the expression of chemokines [6].

In all renal biopsy specimens from IgAN and MesProGN patients focal inflammatory infiltrates in the interstitium were observed and in both studied groups CCR1 and CCR5 immunopositivity was restricted solely to the renal interstitium. The study of Segerer et al. [18] demonstrated that the number of CCR5-positive cells within glomeruli was low, even in cases with proliferative glomerulonephritis. Furuichi et al. [4] detected CCR1 and CCR5-positive cells in both glomeruli and the interstitium in patients with inflammatory renal diseases, including IgAN, and the CCR5-positive cell number was significantly higher in patients with crescentic glomerulonephritis. In our study in the renal tissue from patients with IgAN the number of CCR1+ cells and CCR5+ cells, and the intensity of tubulointerstitial immunostaining of MCP-1 were significantly correlated with the renal interstitial cortical volume, meanwhile in biopsy specimens from patients with MesProGN only interstitial CCR5+ cells were positively correlated with the interstitial cortical volume. Moreover, the mean value of interstitial cortical volume was higher in IgAN patients than in MesProGN group. It is well known that the morphology of IgAN may be identical to that of MesProGN, however approximately 40% of IgAN patients have interstitial fibrosis, mostly focal and mild to moderate in severity [7]. CCR5-positive cells and CCR1-positive cells, which were a prominent part of the interstitial infiltrate may be related to the tubulointerstitial damage in these cases. Vielhauer et al. [20] showed that blockade of the CC chemokine receptor CCR1 reduces interstitial inflammation and fibrosis in murine obstructive nephropathy and progressive nephropathies such as FSGS. Segerer et al. [18] found a significantly higher number of CCR5-positive cells in patient with impaired renal function as compared with patients with normal renal function.

In conclusion, an immunohistochemical comparative study revealed differences in the immunoexpression of chemokine and chemokine receptors in IgAN and MesProGN. Our results suggest that in the renal tissue in IgAN patients MCP-1, RANTES and CCR5 are up-regulated as compared with the renal biopsy specimens in patients with MesProGN. Moreover, MCP-1 as well as CCR5 and CCR1-positive cells may play a role in the interstitial processes leading to fibrosis in IgAN, meanwhile CCR5-positive cells may participate in the interstitial lesions of renal tissue in MesProGN patients.

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