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The Significant Role of RANTES and CCR5 in Progressive Tubulointerstitial Lesions in Lupus Nephropathy

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Glomerulonephritis is an important cause of end-stage renal failure, yet the pathogenetic mechanisms of most forms of glomerulonephritis are not clear. Renal fibrosis is the final common pathway for many kidney lesions that lead to chronic progressive organ failure. Recent study suggests that chemokines and chemokine receptors are involved in the resolution or progression of renal diseases. In view of the above we detected using immunohistochemistry the expression of RANTES, CCR5+ cells,TGF-β1, α-SMA in renal biopsy specimens in 17 patients with IVG A/C class of lupus nephritis and in 10 normal kidneys. The correlative study was undertaken to evaluate the possible relationships between the immunoexpression of RANTES, the number of CCR5+ cells, the immunoexpression of TGF-β1, α-SMA, the value of interstitial cortical volume and the serum creatinine level in patients with lupus nephropathy. Statistical analysis revealed significant increase in tubulointerstitial RANTES immunoexpression in lupus nephritis as compared to normal controls. In our study CCR5+ cells were detected in the interstitium in tissue samples in patient with lupus nephritis, meanwhile no CCR5+ cells were documented in normal controls. We found a strong positive correlation between tubulointerstitial immunoexpression of RANTES and the number of interstitial CCR5+ cells as well as between immunoexpression of RANTES and the immunoexpression of TGF-β1, α-SMA, renal cortical volume and serum creatinine in patients with lupus nephritis. Moreover, the number of interstitial CCR5+ cells was positively correlated with tubulointerstitial α-SMA immunoexpression and renal cortical volume. In summary, the results suggest that in lupus nephritis RANTES may participate in interstitial lesions via CCR5+ cells.

Introduction

Most chronic human kidney diseases are characterized by progressive loss of renal function. Renal fibrosis is the final common pathway for many kidney lesions that lead to chronic progressive organ failure. Tubulointerstitial injury is an important component in the assessment of renal damage because it correlates well with the decline in renal function and long-term progression. Recent studies revealed that chemokines play major role in the mechanism of leukocyte entry into the kidneys, the activation of leukocytes in diseased kidneys, and are involved in the resolution or progression of renal diseases [2]. Many cell types have the ability to generate chemokines. In the renal tissue these include mesangial, endothelial, tubular epithelial as well as interstitial cells. The chemokine superfamily can be divided into four subgroups (C, CC, CXC and CX3C), based upon the position of cysteine residues in a cysteine motif in their primary aminoacid sequence [12]. The CC chemokine subfamily is considered to be the most important in renal pathology. Chemokines of the CC subgroup are composed of MCP-1 (macrophage chemoattractant protein), MIP-1α (macrophage inflammatory protein) and RANTES (regulated upon activation, and normal T-cell expressed and secreted). RANTES, a 68-aminoacid protein, can be expressed by inflammatory infiltrating cells [11], stimulated fibroblast [19, 20], mesangial cells [21] and tubular epithelial cells [27]. The specificity of the chemokine action is determined by the selective expression of the corresponding receptors on immunocompetent cells. Chemokine receptors are classified according to their ligands into the XCR, CCR, CXCR and CX3CR receptor families [17]. Many chemokine receptors are considered "shared receptors" because in vitro they can bind more than one chemokine. CCR5 is

a main receptor for the CC chemokines RANTES, MIP-1α and MCP-1, which are expressed in inflammatory kidney diseases [2, 4]. Infiltrating macrophages and lymphocytes are major source for proinflammatory and profibrotic cytokines [7]. These inflammatory mononuclear cells stimulate resident fibroblast and tubular epithelial cells into α-SMA positive myofibroblasts that secrete abundant extracellular matrix [15, 23]. The activated fibroblast and tubular epithelial cells are stimulated to produce extracellular matrix mainly by cytokine TGF-β1 which is secreted by inflammatory and injured somatic cells [1]. The fibroblast proliferation and secretion of extracellular matrix proteins leads to widening of the interstitial space and loss of renal function [5, 20].

The aim of the present study was to evaluate the immunoexpression of chemokine RANTES, and chemokine receptor CCR5 in renal biopsy specimens in patients with lupus nephritis. Moreover, the possible relationships between the immunoexpression of RANTES, the number of CCR5+ cells, renal interstitial fibrosis, immunoexpression of TGF-β1, α-SMA, and serum creatinine levels were analyzed.

Subjects and Methods

Patients

Renal tissue biopsies were obtained percutaneously for diagnostic purposes from 17 patients (14 females and 3 males, aged 19–47, mean age=31.4) with lupus nephritis. The duration of SLE before biopsy ranged from 6 months to 12 years, meanwhile the clinical and laboratory presentation of kidney disease ranged from 1 to 14 months. Laboratory data including urinalysis, 24h protein excretion and serum creatinine level were collected from each patient. At the time of biopsy 6 patients presented nephrotic range proteinuria, in 10 patient's proteinuria were more than 2g/24h, and in 1 case proteinuria were up to 2g/24h. Renal function impairment was noted 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. Classification of the histopathological lesions refers to that of the World Health Organization [25]. In all renal biopsy samples the histopathological changes refer to IV G A/C WHO class of lupus nephritis. As a control 10 biopsy specimens of the kidneys removed because of trauma were used. None of the persons from control group were 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: polyclonal goat anti-human RANTES antibody (R&D Systems, dilution: 10μg/ml), monoclonal mouse anti-human CCR5 antibody (R&D Systems, dilution: 5μg/ml), polyclonal rabbit anti-human TGF-β1 antibody (Santa Cruz Lab, sc-146, 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, CTS008) prepared according to the instructions of the manufacturer were used. 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 coversliped. 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 RANTES and TGF-β1 in tubuli and renal interstitium 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.

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 (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 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-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 [26]. The point spacing being 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 as a percentage of the total points counted.

Interstitial CCR5+ 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 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 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 of the tubulointerstitial expression of RANTES, the number of CCR5+ cells, the value of interstitial cortical volume, the tubulointerstitial expression of TGF-β1 and α-SMA, are shown in Table 1. In normal controls the immunoexpression of RANTES was weak in
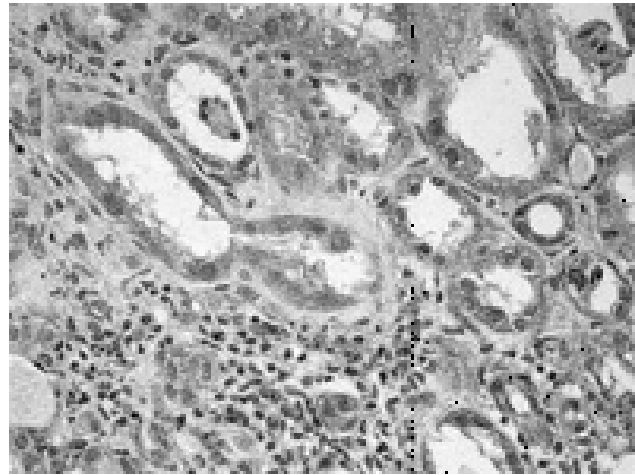

Fig. 1. Lupus nephritis. The immunoexpression of RANTES was moderate to intense in tubular epithelium, interstitium and interstitial infiltrating cells. Magn. × 400.

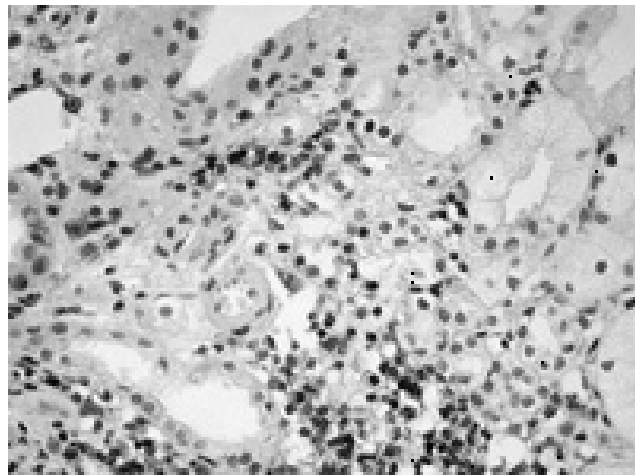Fig. 2. Lupus nephritis. Interstitial CCR5+ cells. Magn. × 400.

proximal tubular epithelial cells and renal interstitial cells. None of the control renal biopsy specimens have evidence of CCR5+ cells and TGF-β1 immunopositivity. In tissue samples in patient with lupus nephritis the immunoexpression of RANTES was moderate to intense in tubular epithelium, interstitium and interstitial infiltrating cells (Fig. 1). The number of interstitial CCR5+ cells was 1.72±1.11 in renal biopsy specimens in patients with

TABLE 1

The tubulointerstitial immunoexpression of RANTES, TGF-β1, α-SMA, the number of CCR5+ cells and value of cortical interstitial volume in renal tissue in patients with lupus nephritis and in normal control

	RANTES	CCR5	TGF-β1	α-SMA	Interstitial cortical volume
Lupus nephritis (n=17)	1.87±0.63	1.72±1.11	1.04±0.76	10.20±9.79	20.93±9.31
Normal control (n=10)	0.05±0.08	0.00	0.00	0.52±0.26	10.91±1.58
P value	P<0.001	P<0.001	P<0.001	P<0.003	P<0.003

Data are expressed as a mean values ±SD

lupus nephropathy (Fig. 2). The immunostainng of RANTES and the number of CCR5+ cells were increased in lupus nephritis as compared with normal control (P<0.001). The immunoexpression of TGF-β1 was detected in the tubular epithelial cells and interstitium in renal biopsy specimens in patients with lupus nephropathy, and the immunoexpression of α-SMA was detected in renal interstitium in both studied groups. In the renal tissue in patients with lupus nephropathy the interstitial immunoexpression of α-SMA was increased as compared with control group (P<0.003). In patients with lupus nephropathy, tubulointerstitial immunostaining of RANTES was significantly correlated with the number of CCR5+ cells (r=0.77, P<0.001), the immunoexpression of TGF-β1 (r=0.53, P<0.03), the immunoexpression of α-SMA (r=0.73, P<0.001), the value of renal cortical volume (r=0.89, P<0.001) and serum creatinine level (r=0.48, P<0.05) (Table 2). Statistical analysis revealed

TABLE 2

The correlations between immunoexpression of RANTES and CCR5+ cells, TGF-β1, α-SMA, interstitial cortical volume, and serum creatinine level in patients with lupus nephritis

Correlations between:	
RANTES and CCR5+	r=0,77, P<0.001
RANTES and TGF-β1	r=0.53, P<0.03
RANTES and α-SMA	r=0.73, P<0.001
RANTES and interstitial cortical volume	r=0.89, P<0.001
RANTES and serum creatinine level	r=0.48, P<0.05

that in renal biopsy specimens in patients with lupus nephropathy the number of CCR5+ cells was positively correlated with the immunoexpression of α-SMA (r=0.57, P<0.02) and the renal cortical volume (r=0.84, P<0.001) (Table 3).

TABLE 3

The correlations between the number of CCR5+cells and the immunoexpression of TGF-β1, α-SMA, interstitial cortical volume and serum creatinine level in patients with lupus nephritis

Correlations between:	
CCR5+ and TGF-β1	r=0.34, P=0.17, NS
CCR5+ and α-SMA	r=0.57, P<0.02
CCR5+ and interstitial cortical volume	r=0.84, P<0.001
CCR5+ and serum creatinine level	r=0.30, P=0.23, NS

Discussion

The involvement of RANTES and their receptor CCR5 in inflammation is well established, but their functional role in disease progression, particularly in the development of fibrosis, is not yet explored. Our study revealed an increase in tubulointerstitial RANTES immunoexpression in renal tissue specimens in patients with lupus nephritis. Moreover, the immunohistochemical staining demonstrated the presence of CCR5+ cells in the interstitium in biopsy specimens in lupus nephropathy. Expression of RANTES has been documented in several human nephritides as well as during experimental glomerulonephritis [6, 11, 14, 18, 19], but to our knowledge the study of this chemokine in lupus nephritis is scanty. Moore et al. [16] showed in MRL-Fas (lpr) mouse, model of systemic lupus, the up-regulation of RANTES. Anders et al. [3] described increased RANTES and CCR5 mRNA during the early phase of apoferritin-induced immune complex nephritis in Balb/c mice. This up-regulation of RANTES preceded the glomerular infiltration of leukocytes. It is presumed that expression of RANTES is significantly lower in patients with non-progressive diseases [15]. Lloyd et al. [14] described that blocking the function of RANTES resulted in a significant decrease of glomerular leukocyte influx and interstitial fibrosis. Recent study suggests that RANTES may participate in a part of renal interstitial lesions via a respective receptor CCR5 [8]. This hypothesis is supported by our results. In our study CCR5+ cells were detected in interstitium in tissue samples from patients with lupus nephritis, meanwhile no CCR5+ cells were documented in normal controls. Similarly to us Furuichi et al. [8] and Wada et al. [24] detected the CCR5+ cells in patients with inflammatory renal diseases including lupus nephritis. Segerer et al. [22] found a prominent expression of CCR5 in the interstitial infiltrate in biopsies of chronic glomerulonephritis and the staining of CCR5 showed the same distribution as CD3+ T cells. It is well known that synthesis of RANTES requires the simultaneous presence of soluble factor and cell-to-cell contact. Haberstron et al. [11] described that contact between T cells and renal tubular epithelial cells, mediated in part by LFA-1/ICAM-1 interaction, is involved in RANTES production. Kuroiwa et al. [13] suggest that RANTES may play a predominant role in migration and activation of T cell, rather than macrophages, on the other hand, T cells infiltrating the interstitium probably activate interstitial macrophages. Activated macrophages, T cells and renal tubular epithelial cells may promote a variety biologic effects, including activation of local cells and amplification of inflammatory responses, induction of renal tubular epithelial cells apoptosis and production of vasoactive mediators, nitric oxide resulting in ischemia and tubular atrophy, as well as transdifferentiation of tubular epithelial cells to fibroblast-like

cells that may promote fibrosis [13]. Our results point to the relationship between RANTES and interstitial lesion in renal tissue. Shown in the present study a strong positive correlation between the tubulointerstitial immunoexpression of RANTES and interstitial cortical volume, tubulointerstitial immunoexpresion of TGF-β1, α-SMA in biopsy specimens in patients with lupus nephritis as well as serum creatinine level suggest the role of RANTES in renal tissue damage. It is thought that CCR5 positive activated interstitial inflammatory cells via RANTES are important in tubulointerstitial dysfunction, possibly by secreting inflammatory mediators. In the present study the number of interstitial CCR5 positive cells was strongly positively correlated with the immunoexpression of α-SMA and renal cortical volume. These results are in concordance with the study of Furuichi et al. [8] who documented that CCR5+cells may play a role in modulating interstitial inflammatory process and tubulointerstitial renal damage. The association of TGF-β1 expression and renal disease progression as well as the profibrotic role of TGF-β1 has been documented in many different human glomerulonephritis [1, 10, 15]. TGF-β1 is a common inducer of transformation of fibroblasts to myofibroblasts, which are major contributors to matrix deposition in tubulointerstital area. It is known that myofibroblasts are terminally differentiated cell with morphologic features between fibroblasts and smooth muscle cells. The cell retains the biologic properties of fibroblasts synthesizing interstitial collagenes I and III, and at the same time, it expresses α-SMA, characteristic of the vascular smooth muscle cell [9, 10]. Similarly to our results, Mezzano et al. [15] described in human membranous nephropathy, a strong up-regulation of RANTES and TGF-β1, which significantly correlates with the degree of tubulointerstitial damage and with the presence of interstitial myofibroblasts. It is noteworthy that myofibroblasts proliferation can be detected before the presence of significant fibrosis, suggesting that they may be potentially used as early markers of progressive disease [7, 9].

In conclusion, our results suggest that RANTES and their cognate receptor CCR5, may mediate progression to fibrosis in lupus nephritis. The strong positive correlations of tubulointerstitial RANTES immunoexpression with infiltrating CCR5+ cells, as well as between RANTES, CCR5+ cells and studied interstitial parameters, strongly support the hypothesis that RANTES is involved in the pathogenesis of renal damage in lupus nephropathy.

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