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Immunohistochemical Analysis of the Interstitial Mast Cells in Rebiopsied Patients with Idiopathic Mesangial Proliferative Glomerulonephritis

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Recent evidence suggests a role for mast cells in the pathogenesis of renal scarring in various glomerulopathies, therefore the present study was undertaken to evaluate whether mast cells have a role in tubulointerstitial fibrosis in rebiopsied patients with idiopathic mesangial proliferative glomerulonephritis (MPG) and to examine a possible relationship between mast cells and interstitial α-smooth muscle actin (α-SMA) expression as well as interstitial infiltrates. Seventeen patients with idiopathic mesangial proliferative glomerulonephritis, in whom renal biopsies were repeated and for whom light and electron microscopy as well as immunofluorescence microscopy and full clinical data were available were examined quantitatively. Morphometric investigations were performed by means of a computer image analysis system. The study revealed that at the rebiopsy proteinuria was significantly lower as compared with the first biopsy. On the other hand, the mean values of the interstitial tryptase positive cells, expression of α-SMA, interstitial volume and CD68+ cells were at rebiopsy significantly increased. The mean values of CD45RB+, CD43+ and CD20+ cells did not differ significantly in these groups. In both initial biopsy and rebiopsy groups there were significant positive correlations between interstitial tryptase positive cells and interstitial expression of α-SMA, interstitial volume, and CD68+ cells. The present quantitative study may suggest that despite of the clinical improvement at rebiopsy, MPG is a progressive glomerular disease, and point to a role of mast cells in this process.

Introduction

Mesangial proliferative glomerulonephritis (MPG) is a morphologic entity that is defined by light microscopy, and is characterized by glomerular mesangial hypercellularity usually affecting all lobules [3], and an inconsistent increase in mesangial matrix. Capillary lumina are patent and capillary walls remain normal [20]. By immunofluorescence glomeruli show mesangial deposition of IgM either alone or as the dominant immunoglobulin with or without complement. Electron dense mesangial deposits are a variable ultrastructural feature [19]. Most of the MPG cases have been associated with the nephrotic syndrome or with persistent proteinuria, while hematuria was reported in a minority of these patients [1, 27]. The prognosis of these idiopathic forms of MPG is uncertain, although some will progress to chronic renal failure [2, 26].

Although semiquantitative and quantitative observations made by light microscopy revealed that glomerular morphology in serially biopsied MPG patients did not correspond to the clinical outcome [4, 5, 28], they proved that the interstitial volume was at the second renal biopsy significantly increased [5].

A great number of cytokines and growth factors produced by the interstitial leukocyte subpopulations are probably involved in tubulointerstitial fibrosis [31]. Recent evidence also suggests a role for mast cells in the pathogenesis of renal scarring in various glomerulopathies [11, 14, 15, 29]. Mast cells contain inflammatory mediators such as histamine, prostaglandin, leukotriens, platelet activating factors and basic fibroblast growth factor [11, 21, 29]. The involvement of mast cells in the process of renal interstitial fibrosis has not been investigated, because these cells are not easily detected by routine histochemical staining [15]. Recently, it is suggested that immunostaining with anti-tryptase monoclonal antibody detects almost all the mast cells in the renal interstitium [11, 16, 30].

Therefore, the present study was undertaken to ascertain whether mast cells have a role in tubulointerstitial fibrosis in rebiopsied patients with MPG and to examine

a relationship between mast cells and interstitial α-smooth muscle actin (α-SMA) expression as well as interstitial infiltrates.

Material and Methods

Patients

Seventeen patients with idiopathic MPG (10 males and 7 females, mean age at the initial biopsy 25.6±10.7) were examined by percutaneous renal biopsy. For the present study only patients in whom repeated renal biopsies were performed were selected. The biopsies were carried out at intervals from 1 to 7.5 years, mean 3.9 years. In all patients indications for renal rebiopsy were relapses after immunosuppressive treatment (as relapses were regarded episodes of recurrent proteinuria or/and hematuria after clinical remission). In each case morphological diagnosis of MPG was established independently by two experienced nephropathologists and based on light microscopy, immunofluorescence and electron microscopy at the time of the first biopsy as well as at the rebiopsy. This study included only cases in which predominant mesangial IgM deposits were seen by immunofluorescence.

Light microscopy

Tissue specimens were embedded in paraffin, sections cut precisely at 4 μm and stained by hematoxylin and eosin, periodic acid-Schiff (PAS)-alcian blue, trichrome light green (Masson), and by silver impregnation (Jones). Thickness of each section was controlled according to the method described by Weibel [32]. For this study biopsies containing less than 10 nonsclerotic glomeruli were neglected.

Immunofluorescence microscopy

A tissue was snap frozen, sectioned at 5 μm and fixed in 95% alcohol for 10 min. Sections incubated with FITC-antisera (Hoechst) to human IgG, IgA, IgM and complement (C3) were viewed in Olympus BX41 microscope, using proper filters.

Electron microscopy

A tissue was fixed in glutaraldehyde, post-fixed in 1% osmium tetroxide, embedded in epon and sectioned on a LKB ultratome. Sections were stained by lead citrate and uranyl acetate, and viewed in a JEM 100B electron microscope.

Immunohistochemistry

The preparation and staining of tissue sections for immunohistochemistry (an indirect StreptABComplex/HRP technique) was carried out as it was described previously [6]. The monoclonal antibodies (DAKO) employed, their specific reactivities and dilutions are listed in the Table 1.

Tissue control for immunostaining

For each MoAb and for each sample a positive control was processed (for mast cells paraffin-embedded sections of a nasal polyp where mast cells were found with toluidine blue staining, paraffin-embedded sections of surgically removed lymph node for interstitial leukocyte infiltrates and vascular smooth muscle cells of the renal biopsy specimens for myofibroblasts). Moreover, the following negative controls were used: 1) omission of primary antibody, 2) incubation with appropriately diluted mouse IgG (DAKO) as a first layer. The samples were prepared by the same method as described above. Specificity of labeling was shown by lack of staining in these samples.

Morphometry

Histological morphometry was performed by means of image analysis system consisting of a IBM-compatible computer equipped with an optical mouse, Indeo Fast card (frame grabber, true-colour, real-time), produced by Indeo (Taiwan), and colour TV camera Panasonic (Japan) linked to a Carl Zeiss Jenaval microscope (Germany). This system was programmed (program MultiScan 8.08, produced by Computer Scanning Systems, Poland) to calculate:

TABLE 1
Monoclonal antibodies employed and their specificity

Monoclonal antibody	Specificity	Source	Dilution
Anti-tryptase (AA-1)	Mast cells	Dako	1 : 100
α-SMA	Myofibroblasts, smooth muscles	Dako	1 : 50
CD45RB	All leukocytes	Dako	1 : 100
CD43+	Pan-T cells	Dako	1 : 100
CD20+	Pan-B cells	Dako	1 : 100
CD68+	Monocytes / macrophages	Dako	1 : 100

– the number of objects (semiautomatic function);
– the surface area of a structure using stereological net (with regulated number of points).

An immunophenotype of mast cells and leukocyte interstitial infiltration was determined by counting all positive cells for each monoclonal antibody (semiautomatic function) in a sequence of ten consecutive computer images of 400 x high power fields – 0.0047 mm^2 each. Mast cells were scored positive when displayed cytoplasmic granules stained positively with anti-tryptase monoclonal antibody. The only adjustments of field were made to avoid glomeruli and large vessels. The results were expressed as a mean number of immunopositive cells per mm^2. Interstitial myofibroblasts were identified by their morphology and positive staining with anti-α-SMA. The interstitial immunoperoxidase staining for α-SMA was measured using point-counting method, which is an adaptation of the principles of Weibel [32], the point spacing being 16 μm. The total 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 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 percent interstitial volume was an expression of the number of points overlying renal cortical interstitium as a percentage of the total points counted.

Statistical methods

Differences between groups were tested using Student's t-test for dependent samples preceded by evaluation of normality and Levene's test. The Wilcoxon matched pairs test was used where appropriate. Correlation coefficients were calculated using Spearman's method. Results were considered statistically significant if $p<0.05$

Results

Clinical and laboratory features of the patients with MPG at the time of a biopsy are given in the Table 2. At the time of the first biopsy 11 patients with MPG showed proteinuria and another 6 nephrotic syndrome. Microhematuria was present in 9 cases; clinical renal impairment (serum creatinine greater than 1.5 mg/dl) was noted in 2 patients and elevated blood pressure in 6. Although at the rebiopsy proteinuria was still present in 12 cases and another 2 were nephrotic, it was significantly lower as compared with the first biopsy (1.75±1.7 and

TABLE 2
The clinical and laboratory data at the time of biopsy in cases with MPG

(n=17)	Micro-hematuria	Gross hematuria	Proteinuria			Nephrotic syndrome	Renal function impairment[1)]	Hypertension (>90/160)
			<1g/24h	1–2 g/24h	2–3.5g/24h			
Initial biopsy	9	–	1	5	5	6	2	6
Rebiopsy	4	1	6	4	2	2	2	4

[1)]Serum creatinine >1.5 mg/dl

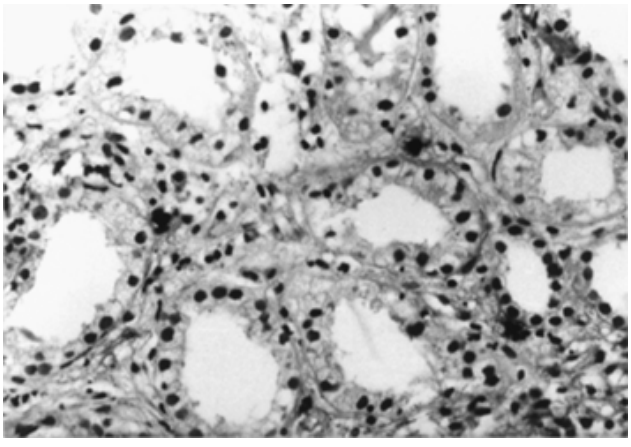

Fig. 1. Immunostaining with anti-tryptase monoclonal antibody of a MPG case at the time of the first biopsy. Some mast cell between tubules can be seen. Magn. 400×.

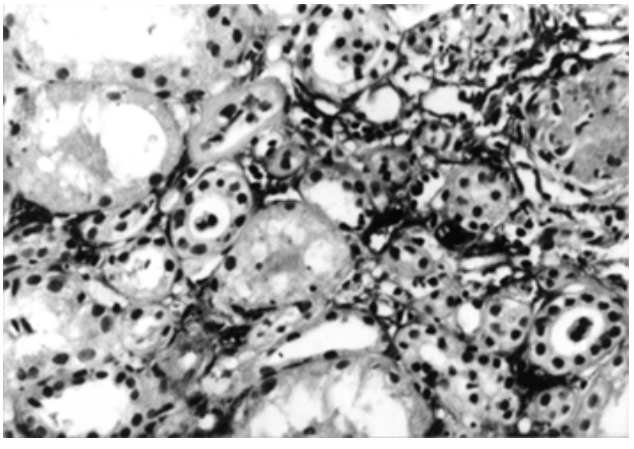

Fig. 2. Immunostaining with anti-tryptase monoclonal antibody in the same patient at rebiopsy. Numerous polygonal in shape mast cells in the area of tubular atrophy. Magn. 400×.

TABLE 3
The morphometric data of interstitial mast cells, expression of α-SMA and interstitial infiltrates in MPG patients at initial biopsy and rebiopsy

Number of cases (n=17)	α-SMA (%)	Interstitial volume (%)	Number of immunopositive cells per 1 mm^2				
			Tryptase+	CD68+	CD45RB+	CD43+	CD20+
Initial biopsy	3.82±0.96	16.23±4.31	7.27±4.16	81.42±36.22	177.31±40.07	84.22±41.15	2.75±1.32
Rebiopsy	6.80±2.04	19.82±5.91	10.79±6.45	99.02±38.27	180.42±41.86	85.14±39.12	2.98±1.41
P value	<0.001	<0.001	<0.001	<0.004	=0.21 (NS)	=0.12 (NS)	=0.28 (NS)

Data are expressed as mean ± standard deviation

TABLE 4
The correlations between tryptase positive cells and interstitial expression of α-SMA, interstitial volume as well as interstitial infiltrates in MPG

Correlation between:	Initial biopsy (n=174)	Rebiopsy (n=17)
interstitial tryptase positive cells and interstitial expression of α-SMA	r=0.61, p<0.01	r=0.57, p<0.02
interstitial tryptase positive cells and interstitial volume	r=0.69, p<0.003	r=0.72, p<0.002
interstitial tryptase positive cells and CD68+ cells	r=0.53, p<0.03	r=0.62, p<0.008
interstitial tryptase positive cells and CD45RB+ cells	r=0.44, p=0.08 (NS)	r=0.25, p=0.33 (NS)
interstitial tryptase positive cells and CD43+ cells	r=0.35, p=0.16 (NS)	r=0.41, p=0.1 (NS)
interstitial tryptase positive cells and CD20+ cells	r=0.22, p=0.57(NS)	r=0.14, p=0.59(NS)

3.26±1.7, respectively, p<0.002). Hematuria was noted in 5 rebiopsied patients, clinical renal impairment (serum creatinine greater than 1.5 mg/dl) in 2 and elevated blood pressure in 4.

The morphometric data of the interstitial tryptase positive cells (Figs. 1 and 2), interstitial expression of α-SMA, interstitial fibrosis and interstitial infiltrates appear from the Table 3. The mean values of the interstitial tryptase positive cells, expression of α-SMA, interstitial volume and CD68+ cells were at a rebiopsy significantly increased in comparison with the initial biopsy. The mean values of CD45RB+, CD43+ and CD20+ cells did not differ significantly in these groups.

The correlations between interstitial tryptase positive cells and interstitial expression of α-SMA, interstitial volume, as well as interstitial infiltrates are shown in the Table 4. In both initial biopsy and rebiopsy groups there were significant positive correlations between interstitial tryptase positive cells and interstitial expression of α-SMA, interstitial volume, and CD68+ cells. The correlations between interstitial tryptase positive cells and CD45+, CD43+ as well as CD20+ cells were positive, but they have not reached statistical significance.

Discussion

The morphometric study showed that in patients with MPG who received immunosuppressive therapy interstitial volume was at rebiopsy significantly increased when compared with the time of the initial biopsy, whereas laboratory data revealed clinical improvement [5]. The present morphometric investigations on interstitial mast cells have provided some interesting insights into the nature of this phenomenon. The idea that mast cells are important to the pathogenesis of renal interstitial fibrosis was recently confirmed in IgA-nephropathy, membranous nephropathy, focal segmental glomerular sclerosis, mesangiocapillary glomerulonephritis, rapidly progressive glomerulonephritis, lupus nephritis and renal amyloidosis [6–9, 11–15, 25]. To our knowledge, however, no data have documented quantitatively interstitial staining for mast cells in serially biopsied patients with MPG.

Our morphometric study showed that in MPG an average number of interstitial tryptase positive mast cells was significantly increased at rebiopsy as compared with the time of the initial biopsy. We observed tryptase positive cells in the interstitium between tubules, around glomeruli and blood vessels. No mast cells were found in the me-

dullary interstitium and in glomeruli. Similar distribution of mast cells was reported in IgA nephropathy, membranous nephropathy, mesangiocapillary glomerulonephritis, lupus nephritis and renal amyloidosis [6–9, 11]. Contrary to our results in proliferative glomerulopathies some mast cells were detected in the medullary interstitium [15]. Likewise in other glomerulopathies [6–9, 11] in the present study the average number of mast cells was lower than the average number of T lymphocytes, but in contrast to the findings of Ehara and Shigematsu [11], the mean number of mast cells was relatively small in comparison with the number of interstitial monocytes/macrophages.

Whereas our results showed that mast cells were one of the constitutive interstitial cell types in MPG, a major finding in this study was the demonstration that in both initial biopsy and rebiopsy groups were significant positive correlations between the number of interstitial mast cells and interstitial volume as well as between interstitial mast cells and interstitial expression of α-SMA. As increased interstitial α-SMA staining can be detected before there appears significant fibrosis [22], it may be speculated that mast cells play a role in the process of myofibroblast stimulation. Although Ehara and Shigematsu [11] suggested that at the present time is not possible decide whether or not mast cell increase as the result of fibrosis, our results are consistent with recent findings of El Nahas group [12] where mast cells infiltration was found to be powerful determinant of interstitial fibrosis. The precise mechanisms of renal interstitial fibrosis and the role of mast cells in this process are not clear [29]. Mast cells have been shown to produce cytokines and growth factors that may contribute to fibrosis [14, 17], whereas heparin and tryptase may enhance fibroblast migration and proliferation [24, 25]. Moreover, fibroblasts and mast cells are known to influence each other [13, 21] and it is suggested that mast cell granule synthesis is also dependent on fibroblasts [10]. Fibroblasts secrete mast cells growth factors, for example c-kit ligand [13]. Recently, El-Nahas group [12] showed using double-labeled immunohistochemistry that expression of the ligand for mast cell c-kit receptor (stem cell factor) highly correlates with mast cell infiltration. These findings suggest that mast cells and its growth factor seem to be up-regulated in glomerulonephritis, and may play a role in the development of renal fibrosis.

On the other hand, we found positive correlations between mast cells and monocytes/macrophages. This relationship may depend on the fact that some tryptase positive cells stained positive with CD68 antibody [11].

Finally, as there is abundant evidence that the extent and severity of interstitial fibrosis and tubular atrophy are the most powerful histological markers of renal function and long term prognosis in chronic glomerulonephritis [23], the present quantitative study may suggest, that despite of the clinical improvement at rebiopsy, MPG is a progressive glomerular disease, and point to the role of mast cells in this process.

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