Expression of macrophage markers in cryoglobulinemic glomerulonephritis – a possible role of CXCL9

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ABSTRACT

Purpose: Cryoglobulinemic glomerulonephritis (CGGN) is a type of membranoproliferative glomerulonephritis (MPGN) that develops in patients with systemic cryoglobulinemia. To date the exact pathogenesis of CGGN remains unclear. It has been suggested that macrophages may be significant contributors to the glomerular injury in this disease. In our study we attempt to characterize the macrophages in human CGGN using classical activation and regulatory macrophage markers.

Material and Method: We searched our database for renal biopsy cases of CGGN. Macrophages were detected using a monoclonal anti-CD68 antibody. Two groups of macrophage markers were used: classical activation markers, including iNOS, CXCL9 and CCL20, and regulatory markers: SPHK1 and LIGHT. The stains were performed using immunohistochemical method.

Results: Five patients with CGGN were identified. Four patients had systemic cryoglobulinemia and two had a serological evidence of hepatitis C virus infection. In all cases the glomeruli contained numerous macrophages. Staining for activatory macrophage markers revealed a strong nuclear staining for CXCL9 in numerous cells, including those corresponding to the macrophage location. Staining for the other activatory markers, as well as staining for regulatory markers, was not significant.

Conclusion: In this study of human CGGN we showed a striking expression of cytokine CXCL9, a classical macrophage activation marker, by the macrophages and possibly other cell types within the glomeruli. This observation points to the possible role of classically activated macrophages in the pathogenesis of MPGN. If this observation is confirmed on a larger group of patients, the cytokine CXCL9 could become a potential therapeutic target for human CGGN.

Key words: Cryoglobulinemia, cryoglobulinemic glomerulonephritis, macrophages, CXCL9.

INTRODUCTION

Cryoglobulinemic glomerulonephritis (CGGN) is a type of membranoproliferative glomerulonephritis (MPGN) that develops due to glomerular deposition of immune complexes in patients with systemic cryoglobulinemia (CG). Most commonly the CGGN is associated with hepatitis C infection [1,2] and contributes to the significant impairment of renal function [3].

Based on experimental animal models it was shown that the immune complex mediated injury in MPGN is multifarious and involves number of pathways, such as activation of complement cascade [4,5], engagement of Fc receptors for immunoglobulins, [6] growth factors (PDGF, TGF-ß) [7] and regulators of renal fibrosing injury (such as TGF-ß and regulators of plasmin: PN-1, tPA, PAI-1 [8-10] ). It is also still not clear what is a major effector cell involved in this type of disease process. The inflammatory infiltrate
in CGGN is mainly composed of macrophages, pointing to this type of cell as being responsible for the tissue response [11]. While macrophages and monocytes are often present in various forms of glomerulonephritis, their exact role remains unknown. It is uncertain whether they are effectors of injury, play beneficial role in the repair, or maybe both.

In respond to injury or infection macrophages are activated in numbers of ways. They respond to various stimuli produced by innate immune cells or antigen-specific immune cells. Additionally, macrophages themselves can generate several factors that influence their own utility. Depending on the type of stimuli macrophages can differentiate into various populations with distinct physiology. Depending on one of the proposals for classification of macrophages they are divided into three groups based on their function: host defense (classically activated macrophages), wound healing (wound healing macrophages) and immune regulation (regulatory macrophages) [12]. All of them develop in response to innate or adaptive stimuli. The classically activated macrophages are produced during cell-mediated immune responses and transiently in response to innate stimuli following stress or infection. The main inductors are interferon-γ (IFNγ) and tumor-necrosis factor (TNF). Activation of this population results in production and secretion of pro-inflammatory cytokines and other mediators of inflammation that can lead to host-tissue damage. Wound-healing macrophages are produced in response to IL-4, one of the first innate signals to be released during tissue injury, eg. following infection with fungi, parasites, and other organisms. These cells secrete components of the extracellular matrix, that can potentially lead to fibrosis, and can exert indirect regulatory effects on the immune response, such as suppression the clonal expansion of neighboring lymphocytes. The third group: regulatory macrophages are produced as a response to stress-induced glucocorticoid release following their stimulation with a TLR agonist in the presence of IgG immune complexes.

In the experimental setting using murine model of immune complex-mediated glomerulonephritis with features of CGGN (thymic stromal lymphopoetin transgenic mice, TSLP-tg, with systemic CG [13]) the conditional ablation of mouse monocytes/macrophages resulted in amelioration of renal injury, suggesting that macrophages are important contributors of tissue injury and have predominately harmful role in the mediation of glomerular injury [11].

In this study we make an attempt to characterize the phenotype of macrophages in human cryoglobulinemic glomerulonephritis using classical activation and regulatory markers. We show for the first time a significant expression of chemokine ligand 9 (CXCL9) that points to the role of the activatory macrophages in the pathogenesis of cryoglobulinemic glomerulonephritis.

MATERIALS AND METHODS

Patient identification

Following the approval from the Institutional Review Board of the Medical University of Bialystok we searched the database of the Department of Pathology for patients who underwent native kidney biopsy between January 2010 and December 2012 and were diagnosed with cryoglobulinemic glomerulonephritis.

The diagnosis of CGGN was established based on the presence of membranoproliferative pattern of glomerular injury, characterized by hypercellular and accentuated lobular architecture due to mesangial and endothelial cell proliferation and influx of leukocytes, glomerular capillary wall thickening or duplication of capillary basement membrane (Fig. 1A), deposition of IgG and/or IgM immune complexes (on immunofluorescence examination, IF) with or without light chain restriction, deposition of complement fragment C3, and preferably organized, subendothelial, luminal and/or mesangial electron dense deposits on ultrastructural examination. The classical cryoglobulins (CGs) appeared as well organized tubules with >30nm in diameter and central hole.

All renal biopsies were processed by standard techniques for light (LM), immunofluorescence (IF) and electron (EM) microscopy. For LM evaluation 2µm histologic sections prepared from formalin-fixed and paraffin embedded tissue were stained with hematoxylin and eosin, periodic acid-Schiff reagent (PAS), and methenamine silver stain. For IF studies, 3µm cryostat sections were stained with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG, IgM, IgA, C3, kappa and lambda light chain, fibrinogen, and albumin (Dako, Carpinteria, CA), as previously described [14]. The distribution of staining was described, and intensity of staining was recorded using a semiquantitative 0 to 4+ score [14]. Tissue for EM was fixed in ½ strength Karnovsky’s solution, postfixed in 2% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in propylene oxide resin. 0.1µm sections were stained with uranyl acetate and lead citrate and examined with Zeiss electron microscope (Germany).

Immunohistochemistry

Formalin-fixed, paraffin embedded tissue sections were processed for immunohistochemistry according to the standard protocols. Macrophages were detected using monoclonal anti-CD68 antibody and lymphocytes were detected by CD3. Macrophages were tested for the expression of activatory markers including iNOS, MIG (CXCL9), and CCL20 (MIP4 alpha) and regulatory markers: SPHK1 and LIGHT. The characteristics of antibodies, including source and dilution used are listed in the Tab. I.
Figure 1. A) Cryoglobulinemic glomerulonephritis with mesangial hypercellularity, influx of leukocytes, double contour of capillary basement membrane and intracapillary immune complexes. B) Numerous macrophages infiltrating the glomerulus as showed by CD68 staining. C) Occasional CD3 positive lymphocytes within the glomerular tuft. D) Strong expression of CXCL9 in the nuclei of numerous cells including macrophages and mesangial cells.

Table 1. Source and dilutions of primary antibodies.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution of primary antibody</th>
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<tbody>
<tr>
<td>CD68</td>
<td>Monoclonal Mouse Anti-Human CD68, DakoCytomation, Glostrup Denmark</td>
</tr>
<tr>
<td>CD3</td>
<td>Monoclonal Mouse Anti-Human CD68, DakoCytomation, Glostrup Denmark</td>
</tr>
<tr>
<td>iNOS</td>
<td>Rabbit polyclonal to iNOS Abcam Inc., Cambridge UK</td>
</tr>
<tr>
<td>CXCL9</td>
<td>Rabbit polyclonal to MIG Abcam Inc., Cambridge UK</td>
</tr>
<tr>
<td>CCL20</td>
<td>Rabbit polyclonal to Macrophage Inflammatory Protein 3 alpha Abcam Inc., Cambridge UK</td>
</tr>
<tr>
<td>SPHK1</td>
<td>Rabbit polyclonal to SPHK1</td>
</tr>
<tr>
<td>LIGHT</td>
<td>Mouse monoclonal to LIGHT Abcam Inc., Cambridge UK</td>
</tr>
</tbody>
</table>
The 2-µm tissue sections were deparaffinized in xylene and rehydrated in graded ethanol. Antigen retrieval was performed by heating tissue sections in antigen unmasking solution (Vector Laboratories, Burlingame, CA). Endogenous peroxidases were blocked in 3% hydrogen peroxide and endogenous biotin was blocked using the avidin/biotin blocking kit from Vector Laboratories. Slides then were incubated with the primary antibody diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Sigma, St. Louis, MO) for 1 hour at room temperature. The sections then were washed repeatedly and incubated with the appropriate secondary antibody. The ABC-Elite reagent (Vector Laboratories) was used for signal amplification, and 3,3-diaminobenzidine with nickel enhancement was used as chromogen, resulting in black color product. Slides were counterstained in methyl green, dehydrated, and coverslipped.

The results of the immunohistochemical staining were reported using a semiquantitative score that ranged from 0 to 3 as follows: 0=no staining detected, 1=< 5 positive cells per glomerulus (in average), 2=between 6 and 10 positive cells per glomerulus (in average), 3=more than 10 cells per glomerulus (in average).

Clinical data
Clinical characteristics determined for each patient included: age, ethnicity, sex, clinical presentation, type of cryoglobulinemia, serology for HCV and HBV, serum creatinine at presentation, eGFR, and proteinuria.

RESULTS
During the review period 371 native biopsies were accessioned. Among these biopsies we identified 4 (1.1%) cases of CGGN. One additional case was submitted by Dr K.O. from the Jagiellonian University. The clinical data and renal biopsy findings are summarized in Tab. 2.

Clinical and pathologic findings
Patient 1
Patient 1 was a 61-year-old male who presented with nephrotic syndrome and hypertension. The laboratory data was significant for elevated serum creatinine (scr) level 2.38 mg/dl, proteinuria 5.5 g/24h, eGFR 28 mL/min/1.73m², low levels of complement component C3 and C4, and positive cryoglobulins. Serological studies were negative for HCV, and c- and p-ANCA antibodies. Kidney biopsy consisted of renal cortex and medulla containing 6 glomeruli for LM, 7 glomeruli for IF and 7 glomeruli for EM. All the glomeruli showed features of MPGN with and cellular crescents involving 30% of glomeruli. Immunofluorescence studies showed granular capillary wall deposits of IgG (1+), IgA (1+), IgM (trace), C3 (1+), and kappa and lambda light chains (each 1+). Ultrastructural examinations confirmed membranoproliferative pattern of glomerular injury with influx of leukocytes and revealed predominately subendothelial immune type electron dense deposits with microtubular substructure.

Table 2. Clinical data and renal biopsy results.

<table>
<thead>
<tr>
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<td>M</td>
<td>M</td>
<td>M</td>
<td>K</td>
<td>M</td>
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<td>47</td>
<td>47</td>
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<tr>
<td>scr (mg/dl)</td>
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<td>eGFR (ml/min/1.73m²)</td>
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<td>107</td>
<td>13</td>
<td>65</td>
<td>35</td>
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<tr>
<td>prot (g/24h)</td>
<td>5.5</td>
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<td>0.22</td>
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<td>pos</td>
<td>neg</td>
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<td>yes</td>
<td>yes</td>
<td>yes</td>
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<td>kid.bx</td>
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<td>MPGN</td>
<td>MPGN</td>
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<td>IF</td>
<td>IgG(1+), IgA(1+), IgM (trace), C3 (1+), kappa (1+), lambda (1+)</td>
<td>not performed</td>
<td>IgG(3+), IgM (2+)</td>
<td>IgG(2+), IgM (2+), C3 (1+), kappa (2+), lambda (2+)</td>
<td>IgG(2+), IgM (1+), C3 (1+), kappa (2+), lambda (2+)</td>
</tr>
<tr>
<td>EM</td>
<td>organized subendothelial deposits</td>
<td>organized deposits mesangial and subendothelial</td>
<td>granular partially organized mesangial and subendothelial deposits</td>
<td>organized deposits mesangial and subendothelial deposits</td>
<td>not performed</td>
</tr>
</tbody>
</table>

Patient 2
Patient 2 was a 63-year-old male who presented with nephrotic syndrome and hypertension. The laboratory data showed scr level of 0.74 mg/dl and proteinuria 1.93/24h. Test for cryoglobulins was negative. Serological studies were negative for HCV, HBV, and ANCA antibodies. Kidney biopsy consisted of cortex containing 11 glomeruli for LM and 5 glomeruli for EM. No glomeruli were present in the portion of the biopsy submitted for IF evaluation. All the glomeruli available for examination showed MPGN. Electron microscopy revealed the presence of numerous immune type deposits localized in mesangial areas and in subendothelial space of the capillary walls. The deposits were focally organized into tubular structures characteristic of cryoglobulins.

Patient 3
Patient 3 was a 28-year-old male who also presented with nephrotic syndrome and hypertension. The laboratory data showed elevated scr level 5.7mg/dl, proteinuria 2.5g/24h and eGFR 13mL/min/1.73m², HCV (+) and positive cryoglobulins. Kidney biopsy showed renal cortex containing 15 glomeruli for LM, 12 glomeruli for IF and 11 glomeruli for EM. The glomeruli had a membranoproliferative pattern of injury with cellular or fibrocellular crescents involving approximately 30% of the glomeruli. IF showed strong staining for IgG (3+), IgM (2+), and C3 (3+) (staining for kappa and lambda light chains was not performed) localized in mesangial areas and in the capillary walls. Ultrastructural examinations confirmed membranoproliferative pattern of glomerular injury and abundant mesangial and subendothelial, partially organized, immune type electron dense deposits.

Patient 4
Patient 4 was a 47-year-old female who presented with nephrotic syndrome and hypertension. The laboratory data showed scr level 1.03mg/dl, proteinuria 0.9g/24h and eGFR 65mL/min/1.73m², positive HCV and positive cryoglobulins. Kidney biopsy revealed renal cortex and medulla. There were 8 glomeruli available for LM, 7 glomeruli for IF and 2 glomeruli for EM examination. The glomeruli showed MPGN, global glomerulosclerosis involving 10% of the glomeruli, segmental glomerular scarring involving 10% of the glomeruli, and fibrocellular crescents involving 10% of the glomeruli. IF showed granular, mesangial and capillary wall staining for IgG (2+), IgM (2+), and C3 (1+), and kappa (2+) and lambda (2+) light chains. EM showed changes consistent with membranoproliferative pattern of glomerular injury and abundant mesangial and subendothelial immune type electron dense deposits organized in form of tubular structures.

Patient 5
Patient 5 was a 47-year-old male who presented with nephrotic syndrome and hypertension. His medical history was significant for idiopathic cryoglobulinemia (diagnosed 2 years earlier) without evidence of HCV infection. The laboratory data showed scr level 1.1mg/dl, proteinuria 0.2g/24h, and positive cryoglobulins. Kidney biopsy consisted of renal cortex containing 12 glomeruli for LM and 5 glomeruli for IF. The glomeruli showed membranoproliferative pattern of injury with influx of leukocytes. IF showed granular staining for IgG (1+), IgM (2+), and kappa (1+) light chains in mesangial areas and in the capillary walls. EM was not performed.

Immunohistochemistry results
The summary of immunohistochemical staining results is provided in Tab. 3. Staining with the anti-CD68 antibody revealed a large number of macrophages in the glomeruli of all patients (Fig. 1B); each biopsy received score of 3. The macrophages were also present within the crescents, both cellular and fibrocellular. Only occasional CD3 positive lymphocytes were detected in the glomeruli in all the cases (average score for all the biopsies was 1; Fig. 1C).

Classical activation markers
In all studied cases the CXCL9, a classical macrophage activation marker, was strongly expressed by number of cells in similar distribution as the previously detected macrophages. The staining appeared to be also present in nuclei of other cells within the glomeruli, possibly in mesangial cells. The overall score for all cases was determined as 3 (Fig. 1D).

The expression of two other activation markers iNOS and CCL20 was negative to weak and was seen in patchy distribution (appropriate controls were positive). Assigned scores were 1 and 0 respectively.

Regulatory macrophage markers
Expression of two regulatory markers SPHK1 and LIGHT was negligible. Only occasional weak staining for LIGHT was observed (score 1 for patients 1, 2 and 4; score 0 for patient 3 and 5), while the staining for SPHK1 was largely negative (score 0 for all cases).

Table 3. Results of immunohistochemical staining for macrophages and the macrophage markers.

<table>
<thead>
<tr>
<th></th>
<th>CD68</th>
<th>iNOS</th>
<th>CXCL9</th>
<th>CCL20</th>
<th>LIGHT</th>
<th>SPHK1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Patient 2</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Patient 3</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Patient 4</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Patient 5</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
DISCUSSION

The primary objective of this study was to investigate the expression of classical and regulatory activation makers of macrophages in human CGGN. We showed that the CXCL9, a classical macrophage activation marker, was substantially expressed by the macrophages in CGGN; while the expression of other tested macrophage markers was not significant. Additionally, no significant contribution of T lymphocytes was detected in the studied cases.

Chemokine ligand 9 (CXCL9), also known as monokine induced by gamma interferon (MIG), is a cytokine that belongs to the CXC chemokine family. CXCL9 is a potent T cell chemoreactant produced mainly by macrophages. It is closely related to two other CXC chemokines called CXCL10 and CXCL11, whose genes are located near the gene for CXCL9 on human chromosome 4. CXCL9, CXCL10 and CXCL11 all elicit their chemotactic functions by interacting with the chemokine receptor CXCR3.

CGGN is a type of glomerular disease, which in most cases is associated with HCV infection [2]. In our study only two out of five patients had a serologic evidence of HCV infection at the time of the biopsy. One could speculate that some of the tests could be falsely negative since it is known that in the presence of cryoglobulinemia, the virus may become trapped in the aggregates of cryoglobulins resulting in negative test. HCV is a hepatotropic virus, which by engagement of both innate and specific immunity leads to the development of chronic infection in the majority of infected individuals [15]. If untreated, chronic hepatitis C infection may lead to cirrhosis, hepatocellular carcinoma, and liver failure [16]. It appears that the inflammation in the liver is, at least partially, driven by the interaction between chemokines secreted by infected cells and corresponding chemokine receptors on migrating leukocytes [17,18].

Recent study by Kurelac et al. [19], showed that the CXCL10 concentrations at the time of a rapid viral response (4 weeks) are better predictors of achieving Sustained Virological Response (SVR) compared to baseline levels. Additionally, this study suggested an important role of CXCL9 as a biomarker of SVR in patients with chronic hepatitis C. The contribution of CXCL9 to the disease pathology has been also showed in other viral infections, such as herpes simplex virus-1 [20] and virus encephalitis [21].

Most of the studies of the role of CXCL9 in the kidneys have been devoted to its role in the T cell mediated regulation. Number of studies showed a significant elevation of the levels of CXCL9 and CXCL10 transcripts in the allografts [22] and urine [23-25] of patients with clinically evident and biopsy proven acute rejection, but also in patients with BK nephropathy (BKN) [26]. While these markers cannot be used to differentiate between rejection and BKN, these chemokines might be used to distinguish acute rejection and BKN from other conditions. In one study involving an experimental model of crescentic glomerulonephritis the authors showed that the interaction between IFNgamma and IL-17-induced chemokines: CXCL9 and CCL20 might orchestrate the time-dependent trafficking and function of renal Th1 and Th17 cells [27].

The role of CXCL9 and its receptor CXCR3 was also studied in a murine model of immune complex-mediated glomerulonephritis by Menke et al. [28]. The authors utilized the experimental model of lupus nephritis (nephritotoxic serum nephritis) and showed an amelioration of kidney disease (measured by renal function and histopathology) and decreased number of T cells and activated macrophages in animals deficient in CXCL9 and its receptor CXCR3. They also showed that both, IgG glomerular deposits and antigen-specific IgG in serum were reduced in these mice, suggesting that although CXCR3 and CXCL9 initiate nephritis through cell-mediated events, renal inflammation may be sustained by their regulation of IgG.

Our study shows, for the first time, a significant expression of CXCL9 in human immune-complex mediated glomerulonephritis (cryoglobulinemic glomerulonephritis). Based on previous observations made in animal models [11] it has been suggested that the macrophages play a significant role in the pathogenesis of this type of injury. The overexpression of CXCL9 in macrophages in this disease suggests its possible role in the pathogenesis of glomerular injury in this particular disease process.

CONCLUSIONS

In summary, in this study we showed a marked expression of cytokine CXCL9 by the macrophages and possibly other cell types within the glomeruli of human CGGN. This observation points to the possible role of classically activated macrophages in the pathogenesis of MPGN in the course of systemic cryoglobulinemia. If this observation is confirmed on a larger group of patients, the cytokine CXCL9 could become a potential therapeutic target for human CGGN.

REFERENCES


