Demonstration of Apoptosis-associated Cleavage Products of DNA, Complement Activation Products SC5b-9 and C3d/dg, and Immune Complexes CIC-C3d, CIC-IgA, and CIC-IgG in the Urine of Patients with Membranous Glomerulonephritis

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Aim. To investigate the involvement of complement activation and apoptosis in the pathogenesis of membranous glomerulonephritis by determining the concentrations of apoptosis-associated 180 bp nucleosomes and complement activation products SC5b-9 and C3d/dg in the urine of patients with membranous glomerulonephritis.

Methods. Morning urine was taken from 15 patients with immunohistologically established membranous glomerulonephritis. Apoptosis-associated 180 bp nucleosomes, complement activation products SC5b-9, C3d/dg, and immune complexes CIC-C3d, CIC-IgA, and CIC-IgG were detected in the urine samples by using antigen-specific enzyme-linked immunosorbent assay.

Results. Concentrations of measured parameters were expressed in units of standard deviation, i.e., relatively to the average concentrations measured in healthy subjects. We found drastically increased concentrations of apoptosis-associated 180 bp nucleosomes (13.71 ± 14.97; p = 0.047), complement activation products SC5b-9 (197.07 ± 134.88; p = 0.003) and C3d/dg (38.70 ± 43.35; p = 0.048), and immune complexes CIC-C3d (11.01 ± 13.39; p = 0.74), CIC-IgA (7.93 ± 4.38; p < 0.001), and CIC-IgG (20.56 ± 10.87; p = 0.001) in the urine of patients with an active form of membranous glomerulonephritis. All studied molecules were absent, or present in very low concentrations, in healthy subjects and patients with membranous glomerulonephritis in remission. The mean differences between healthy controls and patients with the active disease were statistically significant in all parameters, except CIC-C3d.

Conclusions. There is an association of complement activation and apoptosis with membranous glomerulonephritis. Correlation analysis suggests that the excretion of apoptosis-associated 180 bp nucleosomes, SC5b-9, C3d/dg, and immune complexes containing IgA and IgG in the urine of patients with active membranous glomerulonephritis does not depend solely on the passive transport together with other proteins, but is probably an independent active process.

Key words: apoptosis; complement activation; glomerulonephritis, membranous; immunoassay; urine
C3d/dg, and presence of circulating immune complexes CIC-C3d, CIC-IgA, and CIC-IgG in the urine samples.

**Subjects and Methods**

**Patients and Healthy Controls**
Fifteen patients, 11 men and four women, aged 21 to 75 years, were included in the study. All patients had a diagnosis of membranous glomerulonephritis as determined by immunohistological examination of renal tissue samples. In 10 patients, membranous glomerulonephritis was in the active phase and in five patients in remission. The concentration of proteins and the number of erythrocytes and leukocytes were determined in the patients’ morning urine. Activation of the complement system was tested in plasma with CH50 and APH50 tests and by measuring the concentration of complement components C3 and C4. The urine was analyzed for the presence of apoptosis products: 180 bp nucleosomes, complement activation products SC5b-9 and C3d/dg fragment, and circulating immune complexes CIC-C3d, CIC-IgA, and CIC-IgG. Nine healthy persons aged 23 to 50 years were used as control subjects. Urine specimens were collected in ethylene-diamine-tetra-acetic acid (EDTA)-coated test tubes and stored frozen at -70°C until testing.

**Detection of Complement Activation by Classical Pathway**
The activation of the complement classical pathway (CH50) was measured with the hemolytic assay described by Mayer (17). The concentration of hemoglobin released from the lysed cells into the supernatant was determined spectrophotometrically, and the results were expressed as the percentage of the activity of the standard sample (100%).

**Detection of Complement Activation by Alternative Pathway**
The activation of the alternative pathway of the complement (APH50) was measured with the hemolytic assay described by Joiner et al (18). Rabbit erythrocytes were used as the target cells. The concentration of hemoglobin released from the lysed cells into the supernatant was determined spectrophotometrically, and the results were expressed as the percentage of the activity of the standard sample (100%).

**Enzyme-linked Immunosorbent Assay for SC5b-9 in Urine**
The concentration of SC5b-9 was determined by the modified enzyme-linked immunosorbent assay (ELISA) described by Accardo-Palumbo et al (19). The micromotter plates (Nunc, Wiesbaden, Germany) were coated with mouse monoclonal antibodies against SC5b-9 (Diatec AS, Oslo, Norway) and incubated at 4°C overnight. Excess binding sites were blocked with 1% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS). Then, appropriate dilutions of the samples were pipetted in the microwells and incubated for 60 minutes. Bound SC5b-9 was detected with rabbit anti-C5 antibodies (Dako, Glostrup, Denmark) in the second antibody layer. Horseradish peroxidase-conjugated goat anti-rabbit antibody (Dako) was added thereafter. The reaction was visualized by the addition of 1,2-phenylenediamine dihydrochloride (Dako) and H₂O₂ as a substrate, and stopped by adding 12.5% H₂SO₄. The absorbency was measured at 492/620 nm. The SC5b-9 purified according to Deppisch et al (20) was taken as the standard.

**C3d/dg Determination in Urine**
C3d/dg was determined with the double-decker rocket immunoelectrophoresis as previously described by Brandslund et al (21), using rabbit anti-C3c antibodies (Dako, Hamburg, Germany) in the lower and rabbit anti-C3d antibodies (Dako) in the upper gel.

**Determination of the Apoptosis-associated 180 bp Nucleosomes in Urine by ELISA**
Apoptosis-associated nucleosomes containing specific H2A, H2B, H3, and H4 histone epitopes in urine and plasma samples were determined by Cell Death Detection ELISA (Roche Diagnostics GmbH, Mannheim, Germany). In the first step, anti-histone-specific mouse monoclonal antibodies directed against H2A, H2B, H3, and H4 histone epitopes were fixed adsorptively on the walls of the microwell plate module. In the second incubation step, the 180 bp nucleosomes from the samples were bound via their histone components to the immobilized anti-histone antibodies. In the third incubation step, the anti-DNA-peroxidase re-acted with the DNA-part of the nucleosomes. The amount of peroxidase retained in the immune complex was determined photometrically at 405 nm, by using 2,2-azino-di-3 ethylenbenza-zole sulmonic acid (ABTS, Sigma) as a substrate.

**Detection of Circulating Immune Complexes Containing C3d in Urine**
A solid-phase anti-C3d assay, in which wells of microwell plates were coated with monoclonal anti-human C3d antibodies, was used to estimate levels of circulating immune complexes containing C3d (IPI S.p.A., Catania, Italy). Anti-human IgG conjugated to horseradish peroxidase was used to detect bound immune complexes containing C3d and IgG.

**Detection of Circulating Immune Complexes Containing IgA in Urine**
The levels of circulating immune complexes containing IgA and C3b were estimated by a solid-phase anti-C3b assay in which wells of microwell plates were coated with Fabβ15 fragments of goat anti-human C3b antibodies (IPI S.p.A.). Anti-human IgA conjugated to alkaline phosphatase was used to detect bound immune complexes containing C3b and IgA.

**Determination of Circulating Immune Complexes Containing IgG in Urine**
The levels of circulating immune complexes containing IgG and C3b were estimated by a solid-phase anti-C3b assay in which wells of microwell plates were coated with Fabβ15 fragments of goat anti-human C3b antibodies (IPI S.p.A.). Anti-human IgG conjugated to alkaline phosphatase was used to detect bound immune complexes containing C3b and IgG.

**Statistical Analysis**
Concentrations of apoptosis-associated 180 bp nucleosomes, SC5b-9, C3d/dg, CIC-C3d, CIC-IgA, and CIC-IgG were tested with analysis of variance followed by Games-Howell post-hoc comparisons. A value of p<0.05 was considered statistically significant. Correlations were assessed with the Pearson correlation test.

**Results**
The mean values of all measured parameters, except CIC-C3d, significantly differed among the groups (Table 1). In all cases except CIC-C3d, post-hoc tests proved that the active group differed significantly from the healthy controls (Table 2). The concentration of apoptosis-associated 180 bp nucleosomes in the urine of the patients with the active form of membranous glomerulonephritis was much higher than in the urine of patients in remission (p<0.06). Patients and Healthy Controls
Table 1. Concentrations of apoptosis-associated 180 bp nucleosomes (apoptosis), SC5b-9, C3d/dg, CIC-C3d, CIC-IgA, and CIC-IgG in the urine of patients with membranous glomerulonephritis and controls

<table>
<thead>
<tr>
<th>Patients</th>
<th>Concentration (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>apoptosis</td>
</tr>
<tr>
<td>Disease in remission (n=5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.98±1.65</td>
</tr>
<tr>
<td>Active disease (n=10)</td>
<td>13.71±14.97</td>
</tr>
<tr>
<td>Controls (n=9)</td>
<td>0.27±0.59</td>
</tr>
<tr>
<td>F*</td>
<td>4.00</td>
</tr>
<tr>
<td>p</td>
<td>0.037</td>
</tr>
</tbody>
</table>

*ANOVA and Games-Howell post-hoc test.

Table 2. Multiple comparisons of concentrations of apoptosis-associated 180 bp nucleosomes (apoptosis), SC5b-9, C3d/dg, CIC-C3d, CIC-IgA, and CIC-IgG in urine of patients with membranous glomerulonephritis and healthy controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Comparison group vs group*</th>
<th>95% confidence interval for difference</th>
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<tr>
<td></td>
<td></td>
<td>lower limit</td>
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<tr>
<td>Apoptosis</td>
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<tr>
<td></td>
<td>1 3</td>
<td>-1.84</td>
</tr>
<tr>
<td></td>
<td>2 3</td>
<td>0.22</td>
</tr>
<tr>
<td>SC5b-9</td>
<td>1 2</td>
<td>-315.75</td>
</tr>
<tr>
<td></td>
<td>1 3</td>
<td>-0.70</td>
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<td></td>
<td>2 3</td>
<td>77.57</td>
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<tr>
<td>C3d/dg</td>
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<td>-72.61</td>
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<td></td>
<td>1 3</td>
<td>-19.57</td>
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<tr>
<td></td>
<td>2 3</td>
<td>0.34</td>
</tr>
<tr>
<td>CIC-C3d</td>
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<td>-21.77</td>
</tr>
<tr>
<td></td>
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<td>-0.85</td>
</tr>
<tr>
<td></td>
<td>2 3</td>
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</tr>
<tr>
<td>CIC-IgA</td>
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</tr>
<tr>
<td></td>
<td>1 3</td>
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</tr>
<tr>
<td></td>
<td>2 3</td>
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</tr>
<tr>
<td>CIC-IgG</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>2 3</td>
<td>10.39</td>
</tr>
</tbody>
</table>

*ANOVA and Games-Howell post-hoc test.

In conclusion, we confirmed that SC5b-9 binds to glomerular epithelial cells in a receptor-independent manner. This binding causes transcellular vesicular transport of the complex, followed by its exocytosis into the urine (15,29,30). In addition, some authors suppose that SC5b-9 stimulates certain metabolic changes – including membrane lipid turnover, influx of calcium, activation of protein kinase C, the synthesis of pro-inflammatory cytokines, growth factors, and the generation of reactive oxidants in kidney cells (10,12,29,31-33). These changes can then induce changes in the properties of the glomerular basement membrane and subsequent increase in the permeability for complement activation products and apoptosis cleavage products (1,13,29,34-39).

The involvement of apoptosis in the pathological process of membranous glomerulonephritis has not been studied extensively so far (36,38,40-42). We demonstrated the presence of apoptosis-associated mono- and oligonucleosomes, generated during intranucleosomal degradation of genomic DNA, in the urine of patients suffering from membranous glomerulonephritis. Using the TUNEL method, which detects DNA fragmentation with TdT-mediated dUTP nick-end labeling, apoptotic cells were found in renal biopsy material of patients with other diseases affecting kidneys, such as lupus nephritis and IgA nephropathy (16,43-46). The factors necessary for the glomerular cells to enter the apoptotic pathway are not entirely understood. It seems, however, that altered extracellular matrix components play an important role in controlling cell survival and death by influencing apoptotic stimuli (47,48). The role of infiltrating cytotoxic T lymphocytes and natural killer cells in mediating the apoptotic pathway should also be considered (49-51). There are reports of SC5b-9 complex participating in the induction of the apoptotic process, based on the study of experimental glomerulonephritis in rats, where the SC5b-9 played a substantial role in the induction of apoptosis (38,41). The influx of calcium, mediated by SC5b-9, could also contribute to the induction of apoptosis in glomerular cells, since the intracellular increase of calcium acts as a signal for the induction of apoptosis (52).

In conclusion, we confirmed that SC5b-9 and C3d/dg, together with circulating immune complexes of C3d, IgA, and IgG, are present in the urine of patients with membranous glomerulonephritis. We further demonstrated the presence of 180 bp nucleosomes associated with apoptotic disintegration of DNA in the urine of patients with active form of membranous glomerulonephritis. All these products are almost completely absent from the urine of healthy.
people and patients with membranous glomerulonephritis in remission. An important observation is that increased excretion of apoptosis-associated 180 bp nucleosomes, SC5b-9, and C3d/dg does not correlate with proteinuria. This presents the presence of described molecules in urine does not depend simply on the passive transport together with other proteins, but is an independent, probably active process. The presence of apoptotic products together with complement activation products and immune complexes in the urine of patients affected by membranous glomerulonephritis is suspicious of the involvement of complement activation and apoptosis in arising of glomerular lesions in membranous glomerulonephritis (36-38,41,42).

Acknowledgments

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References


