The aim of the study presented here was to investigate whether, in patients showing immediate graft function after renal transplantation, cold-ischemia and reperfusion lead to damage of the glomerular basement membrane and consequently to a loss of heparan sulfate proteoglycans. Loss of these heparan sulfate proteoglycans is a major cause of proteinuria. Time-dependent changes in urinary excretion rates of heparan sulfate proteoglycans but also of total protein, albumin, low- and high-molecular-weight proteins were analyzed quantitatively and by polyacrylamid-gel-electrophoresis in eight patients. Immediately after renal transplantation, severe proteinuria with an excretion rate of up to 251 ± 108 mg/min was apparent and rapidly declined within 24 h to 4.11 ± 2.60 mg/min. The gel-electrophoretic pattern showed a nonselective glomerular and tubular proteinuria. The excretion rate of heparan sulfate proteoglycan was increased in this initial reperfusion phase (up to 7 h), most probably because of ischemia- and reperfusion-induced damage of the glomerular basement membrane. The initial nonselective glomerular proteinuria disappeared within 48 h, changing to a mild selective glomerular proteinuria. In this second phase (7 to 48 h), lower levels of heparan sulfate proteoglycan excretion were observed (0.54 ± 0.54 μg/min versus 1.66 ± 1.93 μg/min, P < 0.05). However, during the repair process of the glomerular basement membrane, heparan sulfate proteoglycan synthesis de novo, leading to an increasing heparan sulfate proteoglycan content of the glomerular basement membrane. This second phase is paralleled by the change from a nonselective to a selective glomerular proteinuria. In the third phase, when the heparan sulfate proteoglycan content of the glomerular basement membrane normalizes, glomerular proteinuria was abolished in most of the patients.

Key Words: ischemia, heparan sulfate proteoglycan, proteinuria, renal transplantation, glomerular basement membrane

Global ischemia is a frequent and clinically important cause of renal injury. After renal transplantation, ischemia- or reperfusion-induced injury of tubular cells leads to acute renal failure in 30 to 40% of the recipients. However, even if immediate graft function takes place, morphological and functional changes are apparent in tubular and glomerular structures of the nephron (1, 2).

Heparan sulfate proteoglycans are important components of the glomerular basement membrane, playing a key role in their molecular organization and structure (3-7). The presence of these strongly negatively charged molecules is essential for maintenance of the selective permeability of the glomerular basement membrane. Loss of the anionic sites provided by the heparan sulfate proteoglycans is associated with proteinuria and has been observed in a number of nephropathies (8-12).

Recently, we have isolated and characterized a novel, small, basement membrane–associated heparan sulfate proteoglycan from human aorta and kidney. Partial amino acid sequence data clearly show that this heparan sulfate proteoglycan is distinct from the large basement membrane–associated heparan sulfate proteoglycan (perlecan) (13). By the use of specific monoclonal antibodies, we have shown that this heparan sulfate proteoglycan is predominantly located in the glomerular basement membrane, to a lesser extent in the basement membrane of tubuli, but also in the mesangium (Figure 1). Previously we have shown that this small heparan sulfate proteoglycan is excreted into urine in the course of the normal turnover but also in nephropathies (13, and unpublished observations). The excretion of this heparan sulfate proteoglycan is correlated with the heparan sulfate proteoglycan content of the glomerular basement membrane, under conditions where de novo synthesis of heparan sulfate proteoglycan can be excluded.

The aim of the study presented here was to investigate whether a loss of glomerular basement membrane heparan sulfate proteoglycans occurs during ischemia and whether this loss leads to proteinuria in...
patients with immediate graft function after renal transplantation.

METHODS

The protocol of the study was in accordance with the Declaration of Helsinki, and full, informed consent was obtained. Time-dependent changes in proteinuria were quantified in eight patients (mean age, 47 ± 9; cold ischemia, 13.7 ± 8.5 h; time for surgical anastomosis, 35 ± 1 min) with immediate graft function after renal transplantation. Clinical data of the patients are shown in Table 1. Kidneys were preserved with the cold-storage solutions Eurocollins ( Fresenius, Oberursel, Germany; two kidneys) or UW (University of Wisconsin, Wisproc; DuPont Pharma, Bad Homburg, Germany; six kidneys). All patients were treated with a triple immuno suppressive therapy consisting of prednisone (100 mg/day), azathioprine (100 mg/day), and cyclosporine.

The urinary protein excretion rates were determined every hour after vascular anastomosis for 7 h and every 24 h thereafter for 10 days. Urinary samples stabilized by sodium azide were collected from a ureteral splint. Total protein, albumin, heparan sulfate proteoglycan (μg/min), low-molecular-weight (α, -microglobulin, β, -microglobulin), and high-molecular-weight protein (transferrin, immunoglobulin (G, C, and α, -macroglobulin) excretion rates were determined, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of urine samples was performed.

The urinary excretion rates were expressed as protein/minute (mg/min) in the urine samples (14). For comparison, excretion ratios (g/mmol creatinine) were also given (Table 2). Urinary creatinine concentration was determined by an automated Jaffe method (Boehringer, Mannheim, Germany) and total protein was measured by a biuret method (Boehringer, Mannheim, Germany). The molecular weight of urinary proteins was characterized by sensitive silver staining of gels after semiautomated microscale SDS-PAGE (15-16).

TABLE 1. Profile of patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex (M/F)</th>
<th>Number of HLA Matches</th>
<th>Duration of Cold Storage (h)</th>
<th>Duration of Anastomosis (min)</th>
<th>Cold-Storage Solution (EC/UW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47</td>
<td>M</td>
<td>6</td>
<td>12</td>
<td>35</td>
<td>EC</td>
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<tr>
<td>2</td>
<td>50</td>
<td>M</td>
<td>6</td>
<td>15.5</td>
<td>35</td>
<td>UW</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>M</td>
<td>5</td>
<td>21.5</td>
<td>35</td>
<td>UW</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>M</td>
<td>2</td>
<td>22.5</td>
<td>33</td>
<td>UW</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>M</td>
<td>6</td>
<td>14.5</td>
<td>35</td>
<td>UW</td>
</tr>
<tr>
<td>6</td>
<td>39</td>
<td>M</td>
<td>6</td>
<td>19</td>
<td>38</td>
<td>UW</td>
</tr>
<tr>
<td>7</td>
<td>48</td>
<td>F</td>
<td>4</td>
<td>18</td>
<td>35</td>
<td>UW</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>F</td>
<td>4</td>
<td>1</td>
<td>35</td>
<td>EC</td>
</tr>
</tbody>
</table>

a EC, Eurocollins solution; UW, University of Wisconsin solution.
TABLE 2. Urinary excretion ratios of proteins (g/mmol creatinine; median values are given) and of heparan sulfate proteoglycan (mg/mmol creatinine) after renal transplantation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time after reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Total Protein</td>
<td>7.2</td>
</tr>
<tr>
<td>Albumin</td>
<td>5.8</td>
</tr>
<tr>
<td>(\alpha_1)-Microglobulin</td>
<td>0.082</td>
</tr>
<tr>
<td>(\beta_2)-Microglobulin</td>
<td>0.015</td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.48</td>
</tr>
<tr>
<td>(\alpha_2)-Macroglobulin</td>
<td>0.010</td>
</tr>
<tr>
<td>IgG</td>
<td>1.2</td>
</tr>
<tr>
<td>HS-PG</td>
<td>0.114</td>
</tr>
</tbody>
</table>

 IgG, immunoglobulin G; HS-PG, heparan sulfate proteoglycan; n.d., not detectable.

(Phast-system; Pharmacia, Uppsala, Sweden). Albumin, transferrin, IgG, and \(\alpha_2\)-macroglobulin were determined using a nephelometric assay (Beckman, Munich, Germany). \(\alpha_1\)-microglobulin and \(\beta_2\)-microglobulin were measured by a quantitative enzyme immunoassay (Elias, Freiburg, Germany). Monoclonal antibodies were raised against the small heparan sulfate proteoglycan from human aorta in mice according to standard procedures as has been described elsewhere (13). Heparan sulfate proteoglycan concentration in urine samples was measured by a sensitive enzyme immunoassay using one of these specific monoclonal antibodies (1F10/B8) as described (13).

Data are given as a mean value ± SD or median as indicated. The probability of error for comparison of the measured values was calculated using the Wilcoxon signed rank test for paired data and the Mann-Whitney U test for unpaired data. The null hypothesis was rejected when \(P < 0.05\). The curve fitting was performed using a polynomial function (median ± 95% confidence interval). The independence of the different variables was checked by means of a Pearson correlation analysis.

RESULTS

Analysis of Urinary Proteins

Immediately after renal transplantation (1 h after kidney reperfusion), a marked proteinuria was apparent (251 ± 108 mg/min) and declined within the first 24 h of graft function (4.11 ± 2.80 mg/min; Figure 2). Albumin excretion rate (111 ± 129 mg/min), accounting for up to 70% of the total proteinuria, declined in parallel with the total protein excretion rate (\(r = 0.97\); Figure 3). Excretion of high-molecular-weight proteins (transferrin 0.45 ± 1.30 mg/min; IgG 1.37 ± 3.99 mg/min; \(\alpha_2\)-macroglobulin 0.033 ± 0.094 mg/min) was observed in all patients during the initial phase (up to 24 h) and disappeared thereafter in most of the patients. Urinary excretion rates of albumin and high-molecular-weight proteins, such as IgG and transferrin, correlated positively during the observation period (\(r = 0.9\), Figure 4).

To exclude a possible influence of creatinine changes after renal transplantation, the protein excretion rates per minute are given. The urinary excretion rate of low-molecular-weight proteins increased in all patients immediately after renal transplantation (\(\alpha_1\)-microglobulin, 2.0 ± 2.0 mg/min, normal range <0.12 mg/min; \(\beta_2\)-microglobulin, 0.20 ± 0.05 mg/min, normal range <0.003 mg/min). Although the urinary excretion rate of these proteins decreased after 24 h, normal values were not reached during the observation period (Figure 5 and 6). \(\alpha_1\)-microglobulin and \(\beta_2\)-microglobulin excretion rates showed similar courses. The gel-electrophoretic pattern changed from a severe nonselective to a mild selective glomerular and tubular proteinuria within 48 to 72 h. The tubular proteinuria persisted in all patients for 10 days, whereas the initial marked nonselective glomerular proteinuria disappeared after 48 to 72 h.

The duration of cold ischemia correlates positively with the \(\alpha_2\)-macroglobulin excretion rate (\(r = 0.28, P < 0.05\)). No correlation was observed between duration of cold ischemia and the urinary excretion rates of total protein, albumin, transferrin, IgG, \(\alpha_1\)-microglobulin, or \(\beta_2\)-microglobulin. However, it should be noted that seven of eight kidneys were subjected to cold ischemia of more than 10 h (range, 1 to 22.5 h).
Heparan Sulfate Proteoglycan

The heparan sulfate proteoglycan excretion rate was increased (up to 2.68 ± 2.44 μg/min at 2 h) in the initial phase up to 7 h, when compared with the urinary heparan sulfate proteoglycan excretion rate in the course of the normal turnover in healthy subjects (0.07 to 0.50 μg/min) (13). In this first phase, the level of heparan sulfate proteoglycan excretion appears to depend on the duration of cold ischemia (r = 0.23, P < 0.05). In the second phase (7 to 48 h), lower levels of heparan sulfate proteoglycan excretion were found (Figure 6). In the third phase (48 to 196 h), the heparan sulfate proteoglycan excretion rose again to near normal (Figure 6). To exclude a possible influence of creatinine changes after renal transplantation, the heparan sulfate proteoglycan excretion rates per minute were given.

DISCUSSION

Heparan sulfate proteoglycans are major components of the glomerular basement membrane and are essential for the maintenance of their charge-selective permeability (3-7). Loss of the anionic sites provided by these strongly negatively charged molecules leads to proteinuria and has been observed in a number of nephropathies (8-12). Reduced synthesis, enhanced turnover, or degradation of heparan sulfate proteoglycans may contribute to the loss of anionic sites in the glomerular basement membrane. However, the exact mechanism of this process is not well understood. In addition sialoglycoproteins as part of the glycocalix of the podocytes may also contribute to the selective filtration function of the kidney (7).

Recently we have isolated and characterized a novel, small, basement membrane-associated heparan sulfate proteoglycan from human kidney (13). This small heparan sulfate proteoglycan is not related to the large basement membrane-associated heparan sulfate proteoglycan (perlecain), as evidenced by partial amino acid sequence data. The small heparan sulfate proteoglycan is excreted into urine in the course of normal turnover, but also in different kidney diseases (13, and unpublished data). Under defined conditions, in which de novo synthesis of heparan sulfate proteoglycan can be excluded, excretion of the small heparan sulfate proteoglycan into urine correlates positively with the heparan sulfate proteoglycan content of the glomerular basement membrane (13).

The aim of the study presented here was to analyze whether cold-ischemia-induced damage of the transplanted kidney leads to a loss of glomerular basement membrane heparan sulfate proteoglycan. A decreased glomerular basement membrane heparan sulfate proteoglycan would then be a major cause of the pronounced proteinuria observed after reperfusion of the transplanted kidney. Furthermore, this study should answer the question of whether de novo synthesis of heparan sulfate proteoglycan during the repair process could be correlated with the course of proteinuria and/or whether the extent and type of the proteinuria could be attributed to different stages of the repair process.

Only patients with immediate graft function could be included in this study because only in these patients was analysis of urinary proteins and heparan sulfate excretion, which reflect a functional impairment or structural lesions of the glomerular basement membrane, possible. In contrast, in patients with acute renal failure, such an investigation would require serial biopsies.

Despite the rapid normalization of renal function, cold-ischemia-induced structural lesions and a functional impairment of the glomerular basement membrane, as well as of tubular cells, was obvious. The functional impairment of the nephron was investigated by biochemical and electrophoretic characterization of excreted proteins (14-16). Whereas tubular lesions could be detected as an increased excretion of low-molecular-weight proteins such as α1-microglobulin and β2-microglobulin, lesions of the glomerular basement membrane are characterized by hyperalbuminuria and excretion of high molecular weight proteins, such as transferrin, IgG, and α2-macroglobulin. In addition to the biochemical analysis of excreted proteins, the determination of glomerular basement membrane heparan sulfate proteoglycan allowed a direct estimation of damage to the glomerular basement membrane (13) as the heparan sulfate proteoglycan content is correlated to the excretion rate. Whereas this was shown previously under defined steady-state conditions, the frequent determination of the heparan sulfate excretion rate in the study presented here will allow investigators to follow also the course of heparan sulfate synthesis and content of the glomerular basement membrane.

Immediately after renal transplantation (within 1 h after kidney reperfusion), a marked proteinuria was apparent and declined within the first 24 h of graft
function. This was in accordance with in vitro and in vivo kidney perfusion studies after cold storage and renal transplantation (1,17,18). A highly increased excretion of albumin and high-molecular-weight proteins was observed in all patients during the initial phase (up to 24 h) and declined rapidly thereafter in most of the patients.

Comparable results were obtained when the data were analyzed either as protein/creatinine excretion ratios or total protein excretion rates (per minute).

Urinary excretion rate of low-molecular-weight proteins was increased (up to 100-fold) in all patients. Although urinary excretion rates of these proteins decreased after 24 h, normal values were not reached during the observation period (10 days after transplantation). The gel-electrophoretic pattern revealed a change from a severe nonselective to a mild selective glomerular and tubular proteinuria within 48 to 72 h (data not shown).

Analysis of the heparan sulfate proteoglycan excretion revealed a strong increase (up to tenfold) in the initial reperfusion phase (up to 7 h), leading to a decreased content of the glomerular basement membrane heparan sulfate proteoglycan (13), and consecutively to the observed marked proteinuria. Therefore, these data clearly show the damage of the glomerular basement membrane, which is most probably a result of cold ischemia. The increased excretion of heparan
sulfate proteoglycan would suggest that the functional impairment of the glomerular basement membrane is not only a result of a reduced synthesis of glomerular basement membrane components such as heparan sulfate proteoglycans, but also to additional damage by oxygen-derived free radicals or other mechanisms during the cold ischemia or reperfusion phase.

Alternatively, the increased excretion of heparan sulfate proteoglycan could be a result of uremic retention. However, this hypothesis is rather unlikely for several reasons. First, the size of the excreted heparan sulfate proteoglycan is comparable with that isolated from human kidney with a molecular weight distribution of Mr 160,000–30,000 (13, and unpublished results). In contrast, the size of the small heparan sulfate proteoglycan from human aorta and other organs is much larger (Mr > 200,000–80,000). Therefore, in healthy subjects, excretion of this highly negatively charged molecule is effectively prevented by the selective permeability of the intact glomerular basement membrane. Our hypothesis that the small heparan sulfate proteoglycan excreted into urine is derived from the glomerular basement membrane and reflects the normal turnover of this heparan sulfate proteoglycan (13) is supported by experimental data from the rat, in which a high turnover of glomerular basement heparan sulfate proteoglycan was observed with half-lives of 5 to 20 h (20–23). Secondly, because of the transcapillary hydraulic pressure difference, the flow in the glomerular basement membrane is directed to the urinary space. Therefore, a retention of the small heparan sulfate proteoglycan or even a retrograde excretion from the glomerular basement membrane into serum rather than into urine, is quite unlikely. Moreover, in patients with severe nephropathy, the glomerular basement membrane is altered and the content of small heparan sulfate proteoglycan, as shown by immunohistochemistry and measured excretion of the small heparan sulfate proteoglycan is greatly diminished (unpublished results). After renal transplantation, when the glomerular basement membrane is also altered (as indicated by the proteinuria), excretion of the small heparan sulfate proteoglycans will be possible. However, because of the selective permeability of the intact glomerular basement membrane, in healthy subjects the small heparan sulfate proteoglycan, which might be released from basement membranes of aorta and other organs, is not excreted into urine. Therefore, mechanisms other than renal excretion should be responsible for the clearance of this heparan sulfate proteoglycans from the circulation, and serum levels of the small heparan sulfate proteoglycan should be independent of kidney function. Although the catabolism of the small heparan sulfate proteoglycan has not been studied in detail until now, for other proteoglycans and glycosaminoglycans such studies have been performed, indicating that most of the small heparan sulfate proteoglycan will be catabolized by resident cells adjacent to the basement membranes and that heparan sulfate proteoglycan which reaches the general circulation will be rapidly cleared by liver endothelial cells (reviewed in Reference 24).

In the repair phase (which could be assigned to the second phase [7 to 48 h], after a lag phase of several hours, de novo synthesis of heparan sulfate proteoglycan begins leading to an increasing content of heparan sulfate proteoglycan in the glomerular basement membrane. However, because of the relatively low content in the glomerular basement membrane, the heparan sulfate proteoglycan excretion rate is lower in the second phase but rises again after 24 h to near normal levels (in the range of 0.07 to 0.50 μg/min). The increasing content of heparan sulfate proteoglycan is not only paralleled by a decrease in proteinuria but also by the shift from a nonselective to a mild selective proteinuria, which can be concluded from the gel-electrophoretic pattern and from the decline of α1-microglobulin and IgG excretion rates (Table 2). In the third phase (after 48 h), where the heparan sulfate proteoglycan excretion normalizes (indicating a normal content of heparan sulfate proteoglycan in the glomerular basement membrane), glomerular proteinuria was abolished in most of the patients. Tubular damage, however, seems to persist for a longer period because elevated excretion of α1- and β2-microglobulin still persists. An additional potentially tubulotoxic effect of cyclosporin A cannot be excluded by the data presented here. Whether in this late repair phase a temporary elevated production of heparan sulfate proteoglycan occurs—suggested by Figure 7—remains to be clarified.

Our data are in accordance with previous findings indicating the importance of heparan sulfate or heparan sulfate proteoglycans for the selective permeability of the glomerular basement membrane (3,19). In these experiments, degradation of glomerular basement heparan sulfate, for example by in vivo treatment with heparitinase, led to a strong proteinuria.
Taken together, these data indicate that the small heparan sulfate proteoglycan in the reperfusion phase of the kidney is especially important because the glomerular basement membrane exhibits a rapid turnover, with half-lives in the range of 5 to 20 h, when examined by metabolic labeling and immunoprecipitation (21-23,25). Whereas the large basement membrane-associated heparan sulfate proteoglycan was not detectable in urine using an enzyme immunoassay (E. Schleicher, Munich, Germany, personal communication), the small basement membrane-associated heparan sulfate proteoglycan was found in relatively high concentrations in urine (in the range of 0.07 to 0.50 μg/min). Taken together, these data indicate that the small heparan sulfate proteoglycan is responsible for the high turnover of glomerular basement membrane heparan sulfate. A possible function of this high turnover might be the regeneration of the anion exchange capacity of the glomerular basement membrane heparan sulfate proteoglycan, which would otherwise become strongly impaired by cationic proteins in the course of the continuous primary filtration process. This is especially important because the glomerular basement membrane, in contrast to most other basement membranes, is only partially covered by fenestrated endothelial cells and is exposed to large amounts of plasma proteins that may absorb onto the basement membrane. However, after renal transplantation and during the cold-ischemia phase and the lag phase after reperfusion, de novo synthesis of the small heparan sulfate proteoglycan is greatly diminished. In this situation, the high turnover and consecutively high excretion rate of the small heparan sulfate proteoglycan may be a disadvantage, and leads to the observed strong proteinuria. Whether analysis of the excretion rate of the small heparan sulfate proteoglycan will allow a prediction of the long-term kidney function after transplantation has to be analyzed in future studies.

REFERENCES