Nephrotic urine prevents increased rat glomerular albumin permeability induced by serum from the same patient with idiopathic nephrotic syndrome

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Abstract

Background. The putative humoral mediator thought to be involved in the pathogenesis of idiopathic nephrotic syndrome has not yet been identified. However, components exist in normal serum that block the permeability activity of FSGS serum in vitro. The potential of FSGS serum to increase glomerular albumin permeability may result from an imbalance between permeability factors and naturally occurring inhibitors. We hypothesized that this imbalance may be favoured by loss of inhibitory factors in nephrotic urine.

Methods. The study population consisted of seven patients with biopsy-proven FSGS, one with IgM nephropathy, and three with idiopathic nephrotic syndrome without biopsy, from whom frozen serum and dialysed and lyophilized urine samples were available. Glomerular albumin permeability (P_{alb}) was determined from the change in glomerular volume induced by applying oncotic gradients across the basement membrane of normal isolated rat glomeruli preincubated with patient serum, normal control serum, patient serum mixed with an equal volume of urine from the same patient, or patient serum mixed with normal urine. Serum and urine apolipoproteins J and E were measured by dot-blot, utilizing peroxidase-labelled antibodies. The urinary capacity to scavenge oxygen radicals was determined after exposure of isolated glomeruli to superoxide generated by xanthine and xanthine oxidase.

Results. The mean P_{alb} of the patients was markedly elevated at 0.74 ± 0.08. The addition of urine from the same patient significantly reduced P_{alb} (mean 0.15 ± 0.23) in all but one of the patients with FSGS. Normal urine had no inhibitory effect in the 10 patients in which it was tested (mean 0.71 ± 0.09). Serum apo J was slightly decreased and serum apo E was slightly increased compared with controls. Urine levels of both lipoproteins were significantly decreased compared with controls. Urine from FSGS patients effectively neutralized superoxide, whereas normal urine did not.

Conclusions. Nephrotic urine but not normal urine contains components that block increased albumin permeability in isolated rat glomeruli induced by serum from patients with the idiopathic nephrotic syndrome. The inhibitory function of these components, which appear not to include apolipoproteins J and E, may involve scavenging of superoxide as a final common pathway. Loss in the urine from the serum of naturally occurring inhibitors in the initial stages of the disease may propagate proteinuria and glomerular injury.

Keywords: apolipoproteins; FSGS; glomeruli; nephrotic syndrome; permeability factors; urine

Introduction

Several lines of evidence have suggested that a circulating humoral factor is involved in the pathogenesis of focal segmental glomerulosclerosis (FSGS), including (i) the often rapid appearance of proteinuria and renal failure following transplantation in affected patients [1,2], (ii) the efficacy of ex vivo techniques including plasmapheresis and immunoadsorption in reducing proteinuria following recurrence [3–5], (iii) permeability changes induced by patient serum in isolated normal glomeruli [6,7] and in cell culture [8], (iv) the possible placental transmission of permeability factors from mother to child [9] and (v) the resolution of proteinuria when kidneys with histological evidence of FSGS are transplanted into patients with end-stage nephrotic syndrome.
renal disease other than FSGS [10]. Nevertheless, the disease is not easily replicated by direct injection of patient serum in healthy laboratory animals [11]. In fact, normal serum from a variety of species contains factors that block the permeability activity of FSGS serum in vitro [12]. We have recently demonstrated that some apolipoprotein components of the high-density lipoprotein complex in normal serum inhibit the permeability activity of FSGS serum in vitro, and clearing of these apolipoproteins from normal serum by specific antibodies restores the permeability activity of the pathological serum [13]. Thus, the integrity of the glomerular permeability barrier in health and disease may depend on the interplay of various permeability factors and their naturally occurring inhibitors.

The stimulus that upsets the balance of permeability and inhibitory factors and the sequence of events that follows the initial insult remain unknown. We hypothesized that loss of inhibitory factors in nephrotic urine may occur, which would amplify the effect of permeability factors in the production of proteinuria.

**Subjects and methods**

**Test and control sera and urine**

Frozen serum and urine samples were available from seven patients with biopsy-proven FSGS, one with IgM nephropathy, and three with idiopathic nephrotic syndrome without biopsy, all of whom had elevated albumin permeability values (P_{ab}, see below) during prior testing. Demographic and clinical characteristics of the patients are shown in Table 1; the majority of patients were in the paediatric age range. Of the 11 patients studied, nine were males. Serum albumin was <15 g/l in all patients with the exception of patient 3, who had low normal values. Pooled normal serum, 24-hour urine samples were collected from six healthy subjects without healthy renal allograft donors. Twenty-four-hour urine of patient 3, who had low normal values. Pooled normal serum, 24-hour urine samples were collected from six healthy subjects without healthy renal allograft donors. Twenty-four-hour urine samples were collected from six healthy subjects without trace proteinuria, to serve as control.

Table 1: Demographic and clinical characteristics of the study population

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Diagnosis</th>
<th>Steroid use</th>
<th>Creatinine (mg/dl)</th>
<th>Proteinuria (g/24 h)</th>
<th>Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>FSGS</td>
<td>Sensitive</td>
<td>0.9</td>
<td>9.0</td>
<td>CsA</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>FSGS</td>
<td>Resistant</td>
<td>0.9</td>
<td>2.0–3.0</td>
<td>Steroid, CF, PP</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>FSGS</td>
<td>Sensitive</td>
<td>0.7</td>
<td>0.1</td>
<td>CsA</td>
</tr>
<tr>
<td>4</td>
<td>41</td>
<td>FSGS</td>
<td>Sensitive</td>
<td>1.2</td>
<td>4.5</td>
<td>CsA, steroid</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>FSGS</td>
<td>Sensitive</td>
<td>2.8</td>
<td>1.8</td>
<td>Steroid</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>FSGS</td>
<td>Sensitive</td>
<td>2.6</td>
<td>2.0–3.0</td>
<td>Steroid</td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>FSGS</td>
<td>Sensitive</td>
<td>0.9</td>
<td>1.0–2.0</td>
<td>CF, PP</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>IgM</td>
<td>Resistant</td>
<td>0.5</td>
<td>4.0–5.0</td>
<td>CsA</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>Idio. NS</td>
<td>Sensitive</td>
<td>0.8</td>
<td>6.0–13.0</td>
<td>CsA, steroid</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>Idio. NS</td>
<td>Resistant</td>
<td>0.5</td>
<td>2.0</td>
<td>Steroid</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>Idio. NS</td>
<td>Resistant</td>
<td>0.3</td>
<td>Trace</td>
<td>CsA</td>
</tr>
</tbody>
</table>

Means ± SD 11.6 ± 10.6 1.10 ± 0.83 3.50 ± 3.21

CsA, cyclosporin; CF, cyclophosphamide; PP, plasmapheresis; Idio. NS, idiopathic nephrotic syndrome.

The protein content was measured, and the volumes equivalent to 100 μg protein were lyophilized (Unicryo MC 2L–60C, Stepbio, Bologna, Italy) and frozen until the time of the experiment.

**Isolation of glomeruli and calculation of P_{ab}**

Glomeruli were isolated from the renal cortex of healthy male Sprague–Dawley rats weighing 200–300 g by sieving in isotonic phosphate-buffer solution. The pH had been titrated to 7.4. The medium also contained bovine serum albumin (BSA) 5 g/dl as an oncotic agent. The isolated glomeruli were then washed in 1 ml of fresh medium, and an aliquot of 0.1 ml was incubated at 37°C for 10 min in 0.9 ml of medium, which included either 2–4% vol/vol patient serum, patient serum with an equal volume of normal urine or nephrotic urine from the same patient, or pooled normal human serum that served as the control. The glomeruli were then plated onto a glass cover slip coated with poly-L-lysine as an adherent, and covered with fresh medium. The samples were masked to eliminate operator bias.

The rationale and methodology for the determination of albumin permeability has been described in detail in the literature [6,14]. In brief, each of 10–16 glomeruli per test serum were videotaped through an inverted microscope before and after a medium change to one containing BSA 1 g/dl. The medium exchange created an oncotic gradient across the basement membrane resulting in a glomerular volume change (\(\Delta V = (V_{final} - V_{initial}) / V_{initial}\)) which was measured off-line by a video-based image analysis program (MCID, Imaging Research Inc., St Catharines, Ontario). The computer program determines the average radius of the glomerulus in two-dimensional space, and the volume is derived from the formula \(V = 4/3\pi r^3\). The magnitude of \(\Delta V\) was related to the albumin reflection coefficient, \(\sigma_{ab}\), by the following equation: \(\sigma_{ab}\) = \(\Delta V\)_{experimental} / \(\Delta V\)_{control}. The \(\sigma_{ab}\) of the control glomeruli was assumed to be equal to 1. \(P_{ab}\) is defined as \((1 - \sigma_{ab})\), and describes the movement of albumin subsequent to water flux. When \(\sigma_{ab}\) is zero, albumin moves across the membrane with the same velocity as water, and \(P_{ab}\) is 1.0. Conversely, when \(\sigma_{ab}\) is 1.0, albumin cannot cross the membrane with water, and \(P_{ab}\) is zero.

**Determination of apolipoprotein J and E levels**

Serum and urinary levels of apo J and apo E were determined by dot-blot, utilizing peroxidase-labelled anti-apo J polyclonal antibodies that upsets the balance of permeability and inhibitory factors and the sequence of events that follows the initial insult remain unknown. We hypothesized that loss of inhibitory factors in nephrotic urine may occur, which would amplify the effect of permeability factors in the production of proteinuria.

Urine specimens were dialysed against water for 48 h using membranes with a molecular-weight cut-off of 8000 kDa.
antibodies (Chemicon, Temecula, CA, USA) and anti-apo E monoclonal antibodies (cell clone 2E11, Roche, Monza, Italy). Apolipoproteins were first adsorbed under vacuum to Hybond C super nitrocellulose (Amersham-Pharmacia, Little Chalfont, UK). Specific antibodies were incubated overnight at room temperature, and visualization was achieved with ECL Plus (Amersham–Pharmacia Biotech, Milan, Italy). Apo J was purified for the standard titration curve according to the method described by Calero et al. [15]. Standards for apo E were purchased from Dauchi Pure Chemicals (Tokyo, Japan). The luminescent signal was acquired with a STORM 860 laser scanner (Amersham–Pharmacia Biotech), utilizing 420 nm and 460 nm as excitation and emission, respectively. Normal values for apo J and apo E in serum are represented by the mean value from six healthy subjects.

Urine scavenging capacity

To determine whether oxygen radical scavenging was involved in the urinary inhibitory activity, the responses of isolated glomeruli after exposure to superoxide generated by xanthine and xanthine oxidase were studied after the addition of normal or nephrotic urine, according to the method described by Dileepan et al. [16]. Briefly, isolated glomeruli were incubated in medium containing 0.1 mmol/l xanthine for 5 min at 37°C. Superoxide was generated by the addition of xanthine oxidase 20 U/ml, 10 µl in 1 ml of 5 g/dl BSA, and the incubation continued for 10 min. Parallel incubations were run in which superoxide dismutase 300 U/ml, pooled normal urine, or separate nephrotic urine samples (4% vol/vol) from three patients with FSGS were added to the incubation medium with xanthine/xanthine oxidase.

Statistical analysis

Data are presented as means ± SD. Comparisons of means were performed by Student’s t-test for unpaired data (two-tailed α = 0.05).

Results

Glomerular albumin permeability following incubation with patient serum, patient serum mixed with urine from the same patient, and patient serum mixed with normal urine is shown in Table 2. In all but one case (patient 3) patient urine abrogated the permeability activity of the matched serum, whereas normal urine did not in the 10 cases in which the experiment was performed. It should be noted that the ‘unprotective’ urine of patient 3 was almost protein free at the time of the measurement.

Serum apo J was non-significantly decreased compared with controls (30 ± 13 vs 36 ± 6 mg/dl), and serum apo E was non-significantly increased compared with controls (16 ± 20 vs 5 ± 3 mg/dl). There was no correlation between the degree of proteinuria and serum apolipoprotein levels (r = –0.41). Both apolipoproteins were significantly (P < 0.001) decreased in patient urine compared with controls (apo J, 0.05 ± 0.05 vs 0.71 ± 0.1 mg/dl; apo E 0.0089 ± 0.0105 vs 0.58 ± 0.1 mg/dl).

Isolated glomeruli exposed to xanthine/xanthine oxidase showed increased albumin permeability (P_{ab} 0.64 ± 0.10, median 0.60, range 0.55–0.80). This reaction was abrogated by simultaneous incubation with superoxide dismutase (P_{ab} 0.23 ± 0.21, median 0.29, range 0.00–0.40). Nephrotic urine significantly reduced albumin permeability induced by superoxide exposure (mean P_{ab} 0.17 ± 0.13, median 0.20, range 0.00–0.33; P < 0.001 vs xanthine/xanthine oxidase), whereas pooled normal urine did not (P_{ab} 0.62 ± 0.18, median 0.58, range 0.44–0.85).

Discussion

The present study demonstrated that a component of urine is capable of blocking in vitro the serum permeability activity of the patient with idiopathic nephrotic syndrome from whom the urine sample was obtained, whereas non-nephrotic urine lacked this potential. One FSGS patient, 3, demonstrated elevated in vitro permeability activity, but appeared to be in clinical remission at the time of testing, and P_{ab} did not improve with the addition of autologous urine. Personal observations of permeability activity after successful plasmapheresis for recurrent FSGS demonstrate rapid return of elevated P_{ab}, which subsequently decreases with time. Thus, it may be that patient 3 was indeed in remission at the time of testing, and the autologous urine was, in fact, normal. Patient 11 had

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Serum P_{ab}</th>
<th>Serum + urine P_{ab}</th>
<th>Serum + normal urine P_{ab}</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>FSGS</td>
<td>0.68</td>
<td>0.37</td>
<td>0.66</td>
</tr>
<tr>
<td>2</td>
<td>FSGS</td>
<td>0.75</td>
<td>0.06</td>
<td>0.65</td>
</tr>
<tr>
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<td>FSGS</td>
<td>0.92</td>
<td>0.74</td>
<td>0.73</td>
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<td>0.00</td>
<td>0.94</td>
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<tr>
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<td>0.74</td>
<td>0.00</td>
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</tr>
<tr>
<td>6</td>
<td>FSGS</td>
<td>0.77</td>
<td>0.00</td>
<td>0.77</td>
</tr>
<tr>
<td>7</td>
<td>FSGS</td>
<td>0.66</td>
<td>0.00</td>
<td>0.77</td>
</tr>
<tr>
<td>8</td>
<td>IgM</td>
<td>0.81</td>
<td>0.00</td>
<td>0.60</td>
</tr>
<tr>
<td>9</td>
<td>Idiopathic</td>
<td>0.68</td>
<td>0.11</td>
<td>0.69</td>
</tr>
<tr>
<td>10</td>
<td>Idiopathic</td>
<td>0.67</td>
<td>0.20</td>
<td>0.73</td>
</tr>
<tr>
<td>11</td>
<td>Idiopathic</td>
<td>0.76</td>
<td>0.15</td>
<td>0.69</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>0.74 ± 0.08</td>
<td>0.15 ± 0.23</td>
<td>0.71 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>
trace protein in the urine but, on the basis of hypoalbuminaemia present at the time of testing, may not have been in remission, and thus his or her urine may have been protective. It is important to stress that the quantity of lyophilized urine protein used during the permeability testing was equal for all subjects, regardless of the quantity collected during the 24-h urine samples.

A systematic study of glomerular albumin permeability and steroid sensitivity has yet to be performed. An unbiased FSGS population to ascertain the correlation between permeability and steroid sensitivity has not yet been performed. An unbiased FSGS population to ascertain the correlation between albumin and steroid sensitivity could not be derived from the present study, the subjects of which presented with difficult management problems and with important risk factors for eventual transplantation.

The most likely candidate for the urinary blocking substance would be a protein filtered through the damaged glomerular permeability barrier or secreted by the tubular epithelium, normally restricted to the blood compartment in conditions of health. In addition to albumin, a number of plasma proteins have been shown to be lost in the urine of patients affected with the nephrotic syndrome, and some of these losses may have pathological consequences. For example, antithrombin III concentrations may be greatly reduced in patients with hypoalbuminaemia, probably because of urinary losses [17], which may contribute to the hypercoagulable state characteristic of the nephrotic syndrome. Decreased serum transferrin levels due to urinary losses may lead to a microcytic, hypochromic anaemia resistant to iron therapy [18].

Since apolipoproteins associated with the HDL complex (namely apo J, apo L, apo E2, apo E4 and a fragment of apo A-IV isolated from normal serum) have the ability to block the permeability activity of FSGS serum in vitro [13], we speculated that the presence of these apolipoproteins in nephrotic urine may be responsible for the blocking activity observed in the present study. However, the results of this study demonstrated that urinary levels of the apolipoproteins J and E were, in fact, significantly lower than in control (i.e. non-proteinuric) urine, and there was no correlation between the degree of proteinuria and the presence of apolipoproteins. Thus, it is unlikely that these apolipoproteins are responsible for the protective effects of autologous urine, since their concentrations are actually greater in normal urine. However, the limited number of cases studied at this point does not allow a firm conclusion.

Few studies have addressed lipid and apolipoprotein abnormalities both in serum and urine of patients and laboratory animals with the nephrotic syndrome. To our knowledge, the present study is the first in which apo J levels have been measured in serum and urine from nephrotic patients. Among the investigations of apo E in nephrotic subjects, Shafir et al. [19] found apolipoproteins A-I, A-II, E and traces of C in control rat urine, and apo E and large amounts of apo C in the urine of nephrotic rats previously treated with puromycin aminoglycoside. Apolipoproteins found in nephrotic urine were virtually always complexed with lipids. van Goor and colleagues [20] studied apolipoproteins A-I, A-IV, E, and B in plasma and kidney tissue from nephrotic rats previously treated with puromycin aminoglycoside or adriamycin. Apo A-I and apo B plasma levels were significantly increased compared to controls, whereas apo A-IV and apo E levels were unchanged. The immunoreactivity of apo A-I, apo A-IV and apo E—those apolipoproteins frequently associated with HDL—was increased in glomerular visceral epithelial cells, whereas apo B and E—frequently associated with VLDL—were found principally in the mesangial matrix. It should be noted that rodents have different serum lipoprotein characteristics compared to humans, particularly in that HDL is the major lipid constituent in rodents. Taken together, however, these data suggest a distinct difference in the apolipoprotein profile both in the plasma and in the urine of nephrotic patients and animals when compared with controls.

In our study, nephrotic urine from FSGS patients prevented increased albumin permeability induced by superoxide generated by the action of xanthine oxidase on xanthine, whereas normal urine did not. Reactive oxygen metabolites have been shown to be key mediators of proteinuria in a number of pathological settings, also before significant histological evidence of glomerular damage is observed. Sharma et al. [21] have recently shown that hydroxyl radicals may mediate increased albumin permeability in isolated glomeruli induced by incubation with transforming growth factor β1. Chen et al. [22] found a role for reactive oxygen species in diabetic glomerulopathy, and Gwinner et al. [23] found increased radical generation in the acute phase of puromycin aminonucleoside glomerulopathy. The concentration of reactive oxygen species, and thus any pathophysiological effects that they may have at the cellular level, is determined by the balance between oxidative enzymes and anti-oxidants such as enzymes (superoxide dismutase, catalase, etc.), metal-binding proteins (such as transferrin), vitamins (A, E, C), among others [24]. Thus, nephrotic urine may be endowed with enhanced anti-oxidant capacity, due to loss in the urine of potent oxygen radical scavengers. Further studies are necessary to isolate the protective factors in urine, to determine the anti-oxidant potential of apolipoproteins in the serum, and to determine whether the putative permeability factor in FSGS initiates a cascade of events which terminates in the production of radical oxygen species and proteinuria.

In conclusion, the present study demonstrates that urine from patients with idiopathic nephrotic syndrome blocks the serum permeability activity of the same patient, just as normal serum blocks the permeability activity of FSGS serum. However, apolipoproteins, which appear to be protective in the serum, do not appear to play a role in urinary protection. The scavenging of radical oxygen species may be the mechanism by which protective factors exert their effect. The loss of protective factors in the urine may lead to a vicious cycle, in which the putative serum permeability factor provokes urinary loss of inhibitory factors.
thereby accentuating the permeability defect and eventual glomerular damage.

References


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