CHARACTERIZATION OF NEUTRAL PROTEINASES IN URINE FROM NEPHROTIC RATS WITH A PROLIFERATIVE OR A NON-PROLIFERATIVE GLOMERULAR DISEASE

J C Davin, *M Davies, J M Foidart, J B Foidart, C A Dechenne, P R Mahieu

State University of Liège, Liège, Belgium, *Welsh National School of Medicine, Royal Infirmary, Cardiff, United Kingdom

Summary

An accelerated model of nephrotoxic nephritis has been used to induce a proliferative glomerular disease in A rats, whereas a non-proliferative glomerular disease has been induced in B rats by intraperitoneal injection of aminonucleoside. In proteinuric A and B rats, the 24-hour urinary excretion of neutral proteinases increases in parallel with that of protein, laminin and type IV collagen. Inhibitory studies indicate that the urinary enzymes are serine-type proteinases and metallo-proteinases which degrade basement membrane polypeptides in vitro. The possibility that these enzymes are implicated in the development of the proteinuria in these models of glomerular disease is suggested.

Introduction

The impermeability of the glomerular filtration barrier to anionic proteins is partly due to its electronegative charges [1]. It has been shown [2,3] that sialic acid and glycosaminoglycans play a major role in the restricted transport of polyanions across the glomerular capillary wall. Furthermore, the ability to obtain reliable growths of glomerular cells in culture has allowed the study of the biosynthesis and degradation of glomerular basement membran (GBM) components in vitro: the epithelial and mesangial cells contribute to the biosynthesis of type IV collagen, non-collagenous glycoproteins and glycosaminoglycans [4,5], whereas neutral proteinases generated by cells derived from the glomerular mesangium are capable of degrading GBM glycoproteins [6]. These findings suggest that both mesangial and epithelial cells may be implicated in the physiological turnover of anionic GBM components in vivo. In immunologically-mediated glomerulonephritis, monocytes and neutrophils frequently infiltrate the glomerulus. These cells contain neutral proteinases which extensively degrade GBM at physiological pH [7] and it is possible that these substances are implicated in the damage to the GBM.
The present study firstly compares the urinary neutral proteinase activity and the urinary excretion of GBM components in normal rats and in rats presenting a glomerular disease associated with a massive proteinuria to provide support for the concept that neutral proteinases may play a role in the damage to the GBM. Since neutral proteinases can be generated in vitro by intrinsic glomerular cells as well as by inflammatory cells, we have also studied the urinary excretion of these enzymes during the course of two experimental models of glomerular disease, i.e. a non-proliferative glomerular disease and a glomerular disease associated with an infiltration of glomeruli by mononuclear phagocytes.

Materials and methods

The experimental models of glomerular diseases

The accelerated model of nephrotoxic nephritis was induced by Sprague Dawley rats (group A) as previously described [8]. Briefly, female rats weighing 100–120g were pre-immunized with 1mg of rabbit IgG in 0.5ml of complete Freund’s adjuvant injected intraperitoneally. One week later, the rats received 1mg of rabbit anti-GBM IgG intravenously. This dose was chosen on the basis of preliminary experiments demonstrating that 1–2mg of rabbit anti-GBM IgG did not induce a significant proteinuria in non-immunized rats [8]. A nephrotic syndrome was induced in another group of Sprague Dawley rats (group B) weighing 100–120g by the administration of aminonucleoside of puromycin (Sigma Chemical Co, St Louis, Mo, USA) in a single intraperitoneal injection of 150mg per kg. Animals were sacrificed three to 24 days after administration of aminonucleoside or of anti-GBM immunoglobulin.

Assessment of urinary neutral proteinase activity

Methods described in the present paper have been extensively detailed [7,9]. Briefly, 24-hour urine collections were exhaustively dialysed at 4°C against distilled water, sterilized by filtration through Millipore filters and stored at −20°C until use. Neutral proteolytic activity against azocasein (Azocoll, Calbiochem-Behring Corp, La Jolla, Ca, USA) or 3H-labelled casein was determined on 0.2ml urine samples according to the method described by Starkey [9]. Under the conditions of the assay, the enzyme activity was linear with time and with enzyme concentration. Enzyme activity is expressed in units per millilitre; one unit is defined as the amount of enzyme hydrolysing 1mg of substrate per hour. To identify which enzyme was responsible for the degradation of substrates experiments with various inhibitors of tissue proteinases were undertaken. The following inhibitors were tested: EDTA 2mM or 15mM, cysteine 2mM, soybean trypsin inhibitor (100 and 50μg/ml), aprotonin (500μg and 1mg/ml) and phenylmethyl sulfonylfluoride (1mM). Enzyme activity is then expressed as the percentage of activity in the absence of inhibitors. Finally, the pH optimum of the proteinase activity was determined by varying urine pH over a range of 6.0 to 8.5 and thermostability was assessed by heating urine samples to 60°C for 30 minutes before testing.
To study the effects of neutral proteinases on basement membrane components, \[^{14}\text{C}\]labelled type IV collagen or laminin were prepared as previously described [4]. The purity of the preparations was confirmed by polyacrylamide slab gel electrophoresis [4]. One microgram of labelled basement membrane polypeptides was added to 0.2ml of 0.02M Tris-HCl buffer pH 7.6, containing 10mM CaCl\(_2\), 0.05% NaN\(_3\), penicillin (100U/ml) and streptomycin (100\(\mu\)g/ml). Dialysed urine samples (0.2ml) were then incubated with the labelled basement membrane polypeptides for 48 hours at 37°C. At the end of the incubation, the samples were exhaustively dialysed against distilled water and their radioactivity was counted in a Packard Tricarb Counter after addition of 10ml of Bray’s solution. Controls included both \[^{14}\text{C}\]labelled polypeptides incubated in buffer alone and \[^{14}\text{C}\]labelled material incubated in urine samples supplemented with phenylmethyl sulfonylfluoride (1mM) and EDTA (2mM). Each experiment was done in triplicate. Results are expressed as the percentage of the radioactivity counted in control samples.

Gel chromatography for the determination of apparent molecular weights of urinary proteinases was performed using a 1.5 x 60cm Sephacryl S-200 SF column (Pharmacia, Uppsala, Sweden) [6], and isoelectric points were determined by chromatofocusing using columns packed with PBE\(\quad\text{T}\)M94 (Pharmacia). Elution was achieved with Polybuffer T74 for the pH 4 to 7 and with Polybuffer TM96 for the pH 6 to 9, according to the instructions provided by the company (Pharmacia, Uppsala, Sweden).

**Assessment of proteinuria and of urinary laminin or type IV collagen excretions**

Twenty-four hour urine collections were used. After dialysis against distilled water, proteinuria was measured as previously described [8], whereas urinary laminin and type IV collagen excretions were measured by a solid phase radio-immunoassay [10].

**Results**

**Urinary proteinase activity in A and B rats**

Microscopic examination of the kidneys from A rats revealed a glomerular hypercellularity which was the consequence of (a) an infiltration of mononuclear cells; (b) a proliferation of intrinsic glomerular cells. In B rats, no glomerular hypercellularity was noted. However, a marked proteinuria (>100mg/24hr) occurred in most animals from both groups. In normal rats, the 24-hour urinary excretion of neutral proteinases ranged from 3.4 to 7.8 Units (mean 5.0±0.71; n: 18). In proteinuric rats from groups A (n=21) and B (n=8), this excretion was significantly increased (mean ± SEM: 27.7±5.34 for A rats and 40.81±10.2 Units for B rats) (p<0.01), and ran in parallel with that of protein, laminin and type IV collagen (data not illustrated). This enzymatic activity was maximal in the neutral range, between pH 7.2 and 7.8. Less than 50 per cent of enzymatic activity were indeed detectable below pH 7.0 or above pH 8.0.
Properties of urinary neutral proteinase activity

In A and B rats, as in normal rats, the urinary neutral proteinase activity was present as a latent and an active form (data not shown); the active form exhibited an apparent molecular weight of about 30,000 and an isoelectric point of 9.0 to 9.6, was destroyed by heating at 60°C for 30 minutes, and was found capable of degrading [¹⁴C]-labelled basement membrane polypeptides in vitro (Table I). In normal urine, the neutral proteinase activity was inhibited by

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Degradation in 18 hours (%)*</th>
<th>Normal rats</th>
<th>A rats**</th>
<th>B rats**</th>
</tr>
</thead>
<tbody>
<tr>
<td>[¹⁴C]-collagen (type IV)</td>
<td></td>
<td>15.1±3.2</td>
<td>52.6±4.8</td>
<td>54.4±3.6</td>
</tr>
<tr>
<td>[¹⁴C]- laminin</td>
<td></td>
<td>21.6±2.9</td>
<td>67.7±3.8</td>
<td>69.6±4.1</td>
</tr>
</tbody>
</table>

* Results are expressed as the percentage of laminin or of type IV collagen counted after incubation with control urines (see Methods section). Data are given as the mean ± SEM (n=4)

** A rats: rats injected with anti-GBM IgG; B rats: rats injected with aminonucleoside

soya bean trypsin inhibitor and aprotinin, but not by EDTA and cysteine, suggesting that this enzymatic activity mainly results from serine-type proteinases (Table II). By contrast, the neutral proteinase activity of urine from proteinuric A and B rats was reduced not only by phenylmethyl sulfonylfluoride,

TABLE II. Effect of potential inhibitors on urinary neutral proteinase activity*

<table>
<thead>
<tr>
<th>Agent</th>
<th>Normal rats</th>
<th>Activity (%)</th>
<th>A rats**</th>
<th>B rats**</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EDTA, 2 mM</td>
<td>100</td>
<td>51.6±2.4</td>
<td>49.8±2.6</td>
<td>48.6±3.9</td>
</tr>
<tr>
<td>Cysteine, 2 mM</td>
<td>100</td>
<td>52.8±1.2</td>
<td>49.8±2.6</td>
<td>48.6±3.9</td>
</tr>
<tr>
<td>SBTI, 500μg/ml</td>
<td>22.4±2.8*</td>
<td>49.8±3.9</td>
<td>49.8±2.6</td>
<td>48.6±3.9</td>
</tr>
<tr>
<td>PMS-F, 1 mM</td>
<td>15.1±4.1</td>
<td>47.1±2.8</td>
<td>56.2±4.2</td>
<td>55.2±4.2</td>
</tr>
<tr>
<td>Aprotinin, 1 mg/ml</td>
<td>19.2±1.4</td>
<td>51.8±3.6</td>
<td>52.3±2.9</td>
<td>51.8±3.6</td>
</tr>
<tr>
<td>EDTA, 2 mM and PMS-F, 1 mM</td>
<td>ND***</td>
<td>9.1±0.4</td>
<td>10.1±0.6</td>
<td>9.1±0.4</td>
</tr>
</tbody>
</table>

* Enzymatic activity is expressed as the percentage of activity in the absence of inhibitors. Data are given as mean ± SEM (n=4)

** A rats: rats injected with anti-GBM; B rats: rats injected with aminonucleoside

*** ND: not done

SBT: Soya bean trypsin inhibitor
PMS-F: Phenylmethyl sulfonylfluoride
soya bean trypsin inhibitor or aprotinin, but also by EDTA or cysteine, which indicates the presence of both serine-type proteinases and metalloproteinases (Table II).

Discussion and conclusions

Proteinases through their action on connective tissue components have been implicated in the pathogenesis of various diseases such as rheumatoid arthritis, atherosclerosis and glomerulonephritis [7]. In this work, further evidence is presented for the involvement of proteinases in glomerular diseases associated with a massive proteinuria, since we have observed in two different experimental models of nephrosis that (a) the excretion of urinary proteinases is increased; (b) these urinary proteinases are capable of degrading basement membrane polypeptides in vitro and particularly laminin, a non-collagenous glycoprotein containing sialic acid; (c) the proteinuria is correlated with the urinary excretion of basement membrane components [10]; (d) the proteinuria and the urinary excretion of basement membrane polypeptides are correlated with the urinary neutral proteinase activity.

From our data, it appears that the neutral proteinases of macrophages could contribute to the glomerular damage in glomerulonephritis observed in A rats. However, mononuclear phagocytes are only observed in glomeruli within two to four days after the injection of anti-GBM IgG, although the proteinuria remains pathological 20 days later [8]. Furthermore, it has been shown that the mononuclear phagocytes infiltrating the glomerular structures in this model of glomerular disease stimulate some metabolic properties of mesangial cells in vitro [8]. Accordingly, we suggest that proteinases secreted by ‘stimulated’ intrinsic glomerular cells are also implicated in the glomerular damage during this proliferative glomerular disease. This possibility is in agreement with the observation that during the aminonucleoside nephrosis, the proteinuria is also correlated with the urinary excretion of neutral proteinases, although no inflammatory cells infiltrate the glomeruli.

Concerning the nature of the proteinases responsible for the glomerular damage, it seems reasonable to suggest that the serine-type proteinases of monocytes are probably not the enzymes responsible since they are inhibited in their action on GBM by different plasma protein inhibitors [7]. Although the monocyte proteinases may be released or secreted directly onto the GBM, in such a way that a local excess of enzyme over inhibitor will result, our data strongly suggest that metalloproteinases generated by glomerular mesangial cells also contribute to the glomerular damage and the proteinuria in certain forms of proliferative or non-proliferative kidney diseases.

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References