INTRODUCTION

The fundamental lesion of diabetic nephropathy (DN) is characterized by hypertrophy of the renal cells, thickening of glomerular and tubular basement membranes and accumulation of extracellular matrix (ECM) proteins within the glomerulus and the tubulointerstitium. These alterations play a key role in the progressive course of DN (1). The components of accumulated ECM proteins in DN include the major collagens (type I, III, IV, V, VI), fibronectin (FN), laminin and small leucine rich (SLR) proteoglycans, while heparan sulfate proteoglycans are reduced (2). An increased accumulation of FN in the glomeruli has been demonstrated in obese Zucker rats (3), in streptozotocin (STZ)-induced diabetes (4), and in human DN (5). In renal tissues of the healthy organism the FN gene expression is generally low, but in early DN FN is present in the ECM and basal membranes as well as in plasma and other body fluids (6-8). At the cell surface FN is a ligand of numerous integrin receptors and is involved in cell adhesion, migration, growth and differentiation. It also binds important molecules such as collagen/gelatin, fibrin and heparin (9). Hyperglycemia or high glucose media results in an augmented expression of genes responsible for FN synthesis (10, 11).

In DN the accumulation of extra/intracellular proteins is consequence of imbalance between synthetic and degradative pathways (3). In the turnover of these proteins various proteolytic enzymes are involved such as matrix metalloproteinases (MMPs) (12), serine proteinases (13), and cysteine proteinases (14, 15). While the role of altered MMPs (lowered MMPs-1, MMPs-2, MMPs-9) and enhanced levels of tissue inhibitors of metalloproteinases (TIMPs-1, TIMPs-2) (4, 12) and of plasmin (elevated levels of plasminogen activator inhibitor (PAI-1)) (13) has been described in detail, the contribution of cysteine proteinases in ECM degradation has received little attention (16). For this reason, in the current study we investigated in isolated glomeruli the potential relationship between the accumulated ECM protein FN and cathepsin B activity in diabetic and healthy rats.

Cysteine proteinases are located in the lysosomes and have their optimum activity in an acidic pH range but still show some activity at the neutral pH (17). They are believed to be involved in the intra- and extracellular protein degradation, including the glomerular basement membrane (14, 15). Cysteine proteinases also activate various enzymes, hormones and growth factors and are upregulated in inflammatory diseases such as rheumatoid arthritis, cancer and neurodegenerative diseases (17-19).

The aim of the current study was to determine whether the accumulation of FN in isolated glomeruli of diabetic rats is associated with lower activity of cathepsin B.

STRONG ASSOCIATION BETWEEN FIBRONECTIN ACCUMULATION AND LOWERED CATHEPSIN B ACTIVITY IN GLOMERULI OF DIABETIC RATS

In diabetic nephropathy the progressive accumulation of extracellular matrix (ECM) proteins results from an imbalance between synthetic and degradative pathways. While the role of the different matrix metalloproteinases in the impaired ECM degradation has been studied in detail, the function of lysosomal cysteine proteinases has not received adequate attention. The aim was to investigate a potential relationship between the accumulated ECM protein fibronectin (FN), and cathepsin B activity in isolated glomeruli of diabetic and healthy rats. Twenty male Wistar rats were included: 10 healthy and 10 with streptozotocin-induced diabetes. After 6 weeks, the experiments were terminated. In the homogenates of isolated glomeruli, FN content and cathepsin B activity were measured by ELISA or spectrofluorometry. FN was also analyzed by immunohistochemistry. Diabetic rats showed a significant rise of systolic blood pressure, impaired renal function and an enhanced urinary excretion of albumin, FN and cathepsin B. In the homogenates of the isolated glomeruli the ratios of FN/protein and FN/DNA showed a trend to higher values, while the ratios of cathepsin B/protein and cathepin B/DNA were reduced. The strong positive association between intraglomerular FN content and cathepsin B activity in both groups suggests that this cysteine proteinase contributes to the degradation of the ECM protein FN. The much higher FN content in DN rats associated with an inadequate cathepsin B activity implies the role of an insufficient FN degradation by cathepsin B and other proteinases.

Key words: cathepsin B, diabetic nephropathy, fibronectin, glomerulus, rats, diabetes mellitus, metalloproteinase
MATERIALS AND METHODS

This study was performed in accordance with the Ethical Committee Affairs Experiments on Animals of the Medical University of Warsaw (Opinion No. 5/2006). Twenty male Wistar rats eight-week-old, weighing 180–200 g were initially included. Diabetes was induced in 10 rats by i.v. injection of 60 mg/kg body weight of streptozotocin (Sigma-Aldrich) as previously described (20); 10 rats received placebo, citrate buffer pH 5.0 alone. After one week, blood glucose levels were evaluated. The animals were followed for six weeks. Blood glucose was regularly, every other day, analyzed. When blood glucose exceeded 700 mg/dl, long-acting human insulin at a dose of 0.25–1.0 U/day was administered. In the last week of the experiment, the blood pressure was measured using a pressure sensor APM MK-9301 (MK-Design, USA). At the sixth weeks of the study the rats were housed in metabolic cages for 24 hours in order to collect the urine. The urine was frozen at –70°C and stored for future analysis. At the end of the experiments the animals were anesthetized with 3.6% chloral hydrate; blood was collected and kidneys were removed for immunohistochemical staining and isolation of glomeruli (21). Glomeruli were isolated according to the method of Spiro (21). Afterwards, they were homogenized with homogenizer Labsonic U (B Braun, USA) and used for determination of DNA using bisbenzimide (Hoechst, Frankfurt, Germany) as previously described (22). The homogenates were further used for the determination of proteins (using the BCA assay (bicinchoninic acid) - protein assay reagent (Pierce, Beijerland, Netherlands)) (23) and FN concentration (ELISA) as previously described (24, 25). Cathepsin B activity in the homogenates, urine and plasma were measured fluorometrically using the synthetic substrate Z-Arg-Arg-AMC (N-CO2-L-arginyl-arginine-7-amino-4-methylcoumarin salt, Bachem, Biochemica GmbH, Heidelberg, Germany) as previously described (26).

Immunohistochemical staining was performed in paraffin sections of the kidney using an antibody against FN (Chemicon International, Temecula, California, USA). The analysis started with assessment of the representative areas of the entire tissue section. We evaluated 20 subsequent glomeruli within the renal cortex of representative areas. In relation to the glomerular vascular loops each immunohistochemical reaction assessment was based on an analysis of two features: the intensity of the antibody reaction and the percentage of immunopositive vascular loops. Immunohistochemical expression was evaluated semiquantitatively using a 4-point scale:

0) no reaction or very weak and focal (<1%) expression;
1+) low intensity reaction, with moderate intensity expression of <50% of vascular loops, and high intensity expression being segmental or in <25% of vascular loops;
2+) moderate intensity expression involving >50% of vascular loops and high-intensity expression being segmental or continuous involving 25–75% of vascular loops;
3+) high intensity expression that is continuous and includes >75% of vascular loops;

The final evaluation for FN was the average (rounded to unity) from the analysis of 20 glomeruli.

Table 1. The characteristics studied of the healthy and DM treatment groups. Results are presented as mean ±S.D.

<table>
<thead>
<tr>
<th></th>
<th>Healthy n=9</th>
<th>DM n=7</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td><strong>Initial body weight, g</strong></td>
<td>326.1 ± 32.9</td>
<td>327.0 ± 21.6</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Final body weight, g</strong></td>
<td>354.4 ± 22.0</td>
<td>264.7 ± 54.8</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Kidney weight, g</strong></td>
<td>1.42 ± 0.11</td>
<td>1.57 ± 0.25</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Kidney /body weight ratio, %</strong></td>
<td>0.40 ± 0.03</td>
<td>0.60 ± 0.06</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

**Blood parameters**

<table>
<thead>
<tr>
<th>parameter</th>
<th>Healthy</th>
<th>DM</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Pressure, mmHg</td>
<td>130.45 ± 12.75</td>
<td>173.36 ± 26.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>15.78 ± 2.70</td>
<td>41.43 ± 2.64</td>
<td>0.01</td>
</tr>
<tr>
<td>Sodium, mmol/l</td>
<td>139.6 ± 2.0</td>
<td>135.0 ± 2.2</td>
<td>0.01</td>
</tr>
<tr>
<td>Creatinine, µmol/l</td>
<td>38.94 ± 12.38</td>
<td>58.41 ± 9.73</td>
<td>0.005</td>
</tr>
<tr>
<td>Urea, mmol/l</td>
<td>7.64 ± 1.10</td>
<td>10.42 ± 2.68</td>
<td>0.05</td>
</tr>
<tr>
<td>Total protein, g/l</td>
<td>59.0 ± 4.0</td>
<td>51.0 ± 3.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Fibronectin, mg/l</td>
<td>22.60 ± 4.116</td>
<td>20.91 ± 4.47</td>
<td>NS</td>
</tr>
<tr>
<td>Cathepsin B, µIU/l</td>
<td>48.65 ± 14.72</td>
<td>44.61 ± 11.88</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Urine parameters**

<table>
<thead>
<tr>
<th>parameter</th>
<th>Healthy</th>
<th>DM</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diuresis, ml/h</td>
<td>0.54 ± 0.24</td>
<td>1.38 ± 0.24</td>
<td>0.00001</td>
</tr>
<tr>
<td>Creatinine, µmol/24h/body weight</td>
<td>2.65 ± 0.88</td>
<td>2.57 ± 1.15</td>
<td>NS</td>
</tr>
<tr>
<td>Microalbuminuria, µg/24h/body weight</td>
<td>0.33 ± 0.17</td>
<td>1.65 ± 1.00</td>
<td>0.0001</td>
</tr>
<tr>
<td>Fibronectin, ng/24h/body weight</td>
<td>0.61 ± 0.33</td>
<td>1.10 ± 0.53</td>
<td>0.02</td>
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<tr>
<td>Cathepsin B, µIU/24h/body weight</td>
<td>4.1 ± 2.1</td>
<td>21.2 ± 4.6</td>
<td>0.0002</td>
</tr>
<tr>
<td>Cathepsin B/creatinine, µIU/µmol</td>
<td>11.5 ± 4.4</td>
<td>77.0 ± 25.9</td>
<td>0.00008</td>
</tr>
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</table>

Table 2. Cathepsin B activity and FN concentrations in renal cortex and in glomerular homogenates. Results are presented as mean ±S.D.

<table>
<thead>
<tr>
<th></th>
<th>Healthy n=9</th>
<th>DM n=7</th>
<th>p value</th>
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<tbody>
<tr>
<td><strong>Glomerular homogenates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein/DNA, µg/µg</td>
<td>19.36 ± 5.66</td>
<td>23.2 ± 6.5</td>
<td>NS</td>
</tr>
<tr>
<td>Fibronectin/protein, ng/µg</td>
<td>0.041 ± 0.019</td>
<td>0.054 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Fibronectin/DNA, ng/µg</td>
<td>0.72 ± 0.36</td>
<td>0.95 ± 0.28</td>
<td>NS</td>
</tr>
<tr>
<td>Cathepsin B/protein, µIU/µg</td>
<td>2.57 ± 1.25</td>
<td>1.36 ± 0.37</td>
<td>0.035</td>
</tr>
<tr>
<td>Cathepsin B/DNA, µIU/µmol</td>
<td>40.71 ± 26.30</td>
<td>30.60 ± 9.65</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Immunohistochemistry Renal Cortex Fibronectin**

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>DM</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomeruli</td>
<td>1.22 ± 0.97</td>
<td>2.33 ± 0.52</td>
<td>0.049</td>
</tr>
<tr>
<td>Interstitial tissue</td>
<td>1.11 ± 1.5</td>
<td>1.83 ± 0.41</td>
<td>NS</td>
</tr>
</tbody>
</table>
**Statistical analysis**

Data are given as means ±S.D. Statistical analysis was performed with the nonparametric Mann-Whitney test. Correlation analyses were performed using Pearson’s correlation, p<0.05 was considered statistically significant.

**RESULTS**

Table 1 shows clinical and biochemical characteristics of the rat groups. The diabetic group had significantly higher blood pressure (p=0.001), higher levels of blood glucose (p=0.01), creatinine (p=0.005), and urea (p=0.05), while serum sodium and total protein were significantly lower (p=0.01). Potassium, bicarbonate, cholesterol and triglyceride levels did not change significantly (data not shown). The 24 h urine of diabetic rats showed a significant increase of diuresis (p=0.00001), excretion of microalbumin/body weight (p=0.0001) and FN/body weight (p=0.02), and activity of cathepsin B/body weight (p=0.0002).

Table 2 shows data about body and kidney weight as well as the concentrations of the tested parameters in isolated glomeruli and in the kidney cortex. Compared to controls, the diabetic group displayed an increased kidney/final body weight ratio (p=0.0001), although the body weight declined. In the homogenates of glomeruli, an enhanced protein/DNA ratio (NS), FN/DNA ratio (NS), and FN/protein ratio (NS) was observed. In kidney cortex, staining of FN in the glomeruli by immunohistochemistry showed higher levels in diabetic rats compared to controls (p=0.049). In contrast, the cathepsin B/DNA ratio (NS) and cathepsin B/protein ratio (p=0.035) in homogenates of glomeruli were lower in diabetic rats than in controls.

There was a significant correlation between the intraglomerular FN/protein ratio as well as FN/DNA ratio and the intraglomerular cathepsin B/protein and cathepsin B/DNA ratios in the diabetic and non-diabetic groups (Figs. 1 and 2).

A significant positive correlation was also found between the intraglomerular cathepsin B/DNA ratio and the urinary cathepsin B/creatinine ratio in the diabetic group (p<0.05, r=0.81); no relationship was found in the control group (r= -0.13) (Fig. 3).

Moreover, in the diabetic group a significant correlation existed between glomerular FN content (according to the immunohistochemical staining score) and the cathepsin B/protein ratio in the isolated glomeruli (p<0.05, r=0.92); while no such relationship was observed in the controls (Fig. 4). There was a significant difference in the glomerular FN content of diabetic and control rats (p=0.049) (Fig. 5).

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**Fig. 1.** Correlation of FN/protein and cathepsin B/protein concentrations in homogenates of renal glomeruli. DM - diabetic rats, p<0.05, r=0.88; Control - healthy rats, p<0.05, r=0.84.

**Fig. 2.** Correlation of the concentration of FN/DNA and cathepsin B/DNA in homogenates of glomeruli. DM - diabetic rats, p<0.05, r=0.94; Control - healthy rats, p<0.05, r=0.76.
Fig. 3. Correlation between activity of cathepsin B/DNA in homogenates of glomeruli and cathepsin B/creatinine excreted in the urine. DM - diabetic rats: p <0.05, r = 0.81; Control - healthy rats: no statistical significance.

Fig. 4. Correlation between glomerular content of FN assessed by immunohistochemistry and activity of cathepsin B/protein in glomeruli homogenates. DM - diabetic rats, p<0.05, r=0.92; Control - healthy rats, no significant correlation.

Fig. 5. Immunohistochemistry - fibronectin content within the glomerulus of a healthy rat (A) and diabetic rat (B). 40 magnification, scale 50 µm.
Excessive deposition of ECM proteins in DN is in part a consequence of an inadequate protein degradation. In particular depression of MMPs and higher levels of TIMPs contribute to the impaired protein-turnover (12, 27). Moreover, in DN the altered plasminogen activator/inhibitor system is involved in matrix accumulation. The elevated levels of PAI-1 in DN not only inhibit the serine proteinase but also exert pronounced pro- fibrotic actions (13). In our study in the STZ-induced diabetic rats we demonstrated that the lower intraglomerular cathepsin B activity compared to controls was in line with earlier reports (3, 4, 20, 28). As we expected, the glomerular FN content in the renal cortex was increased as evaluated by immunohistochemical staining and in the homogenates of isolated glomeruli in the diabetic rats compared to non-diabetic controls. Also these results are consistent with previous reports (3, 7, 8). In this study we showed the relationship between accumulated FN and cathepsin B activity in the diabetic and non-diabetic rats, which according to our knowledge a new finding. Moreover, in the diabetic rats, the FN/DNA ratio (as well as FN/protein ratio), depicted against the corresponding cathepsin B activity/DNA ratio was markedly enhanced as compared to the non-diabetic controls. These data support previous investigations (14, 15) which demonstrated that cathepsins are involved in the intra/extra cellular protein degradation, including glomerular basement membranes. In former studies it has also been reported that inhibition of lysosomal cathepsins may suppress the intracelllular protein breakdown by up to 70% (29).

On the other hand, clinical conditions with augmented protein degradation were associated with an enhanced cathepsin B activity (30).

In our investigations the plasma concentrations of cathepsin B and FN tended to lower levels. The latter finding is surprising, since other authors observed a rise of FN in DN (8, 31). We have no explanation for these different observations.

In contrast to the lowered cathepsin B activity in the isolated glomeruli of diabetic rats, the urinary excretion of this enzyme was significantly enhanced, associated with an augmented excretion of albumin and FN. Enhanced urinary cathepsin B levels have also been reported earlier in STZ rats (4) and in diabetic patients (32). Most probably is the enhanced urinary enzyme excretion a consequence of a protein reabsorption overload of the cells of the proximal tubules (16). According to Olbricht et al. (33) cathepsin B and L activities in isolated nephron segments from proteinuric rats are much higher than in non-proteinuric animals. It is conceivable that the urinary losses of this proteinase contribute to the lowered cathepsin B activity in the kidney in DN.

Hyperglycemia is the principle factor for the metabolic and structural alterations in diabetes. The pathways include enhanced formation of reactive oxygen species (ROS), activation of protein kinase C, generation of early and advanced glycation end products (AGEs), as well as transforming growth factor β1, (TGF-β1), activation of the renin angiotensin aldosterone system (RAAS) and mechanical forces due to intraglomerular hypertension (16). The deleterious consequences from AGE-accumulation include enhanced formation of intra/extracellular proteins and a suppressed protein degradation. The plasma levels of various MMPs are lowered, while the synthesis of TIMPs and PAI-1 are enhanced (34). In our investigations incubation of renal tubule cells with AGE-BSA resulted in a lowered cathepsin B activity (35). Moreover, as a consequence of AGE-induced cross-link formation, the susceptibility to protein degradation is reduced. The interaction of AGES with their cellular receptor RAGE induces various signaling cascades with an enhanced formation of NFkB, ROS and TGF-β1 (36). In particular TGF-β1 mediates many AGES effects. In various kidney cells, it enhances the production of ECM proteins such as collagen I and IV, FN and laminin. Furthermore, TGF-β1 suppresses matrix degrading enzymes by inhibition of the MMPs synthesis and an enhanced production of TIMPs and PAI-1. In our investigations in renal tubular cells, TGF-β1 increased the protein synthesis and induced a significant decline of cathepsin B activity (37). Another factor involved in the development of renal fibrosis is the connective tissue growth factor (CTGF). A recent study indicates that this cytokine is a pivotal mediator for TGF-β-induced expression of matrix proteins including FN (38). Among the factors involved in TGF-β1 formation, also the hyperglycemia-induced angiotensin II formation has to be mentioned (39). It suppresses MMPs while enhancing TIMPs and PAI-1 (27).

In the therapy, control of hyperglycemia is of fundamental importance. Administration of ACE inhibitors as well as angiotensin II type 1 receptor blockers attenuate the accumulation of extracellular matrix both in humans and animals suffering from diabetes. In the past several years it has been shown that administration of RAAS blocker are associated with a suppression of TGF-β1 (27).

In previous studies of our group (40) administration of a mixture of trypsin and bromelain, stabilized with rutoside in diabetic rats reduced levels of FN/DNA and growth factor TGF-β1/DNA in diabetic rats as compared to untreated diabetic rats.

Positive effects of these enzymes may be due to cleavage of the external domain of the receptor of AGES (RAGE) with subsequent lowering of TGF-β1 production (41).

Taken together our data suggest that the accumulation of FN in diabetic nephropathy will be in part a consequence of suppressed cathepsin B activity.

Conflict of interests: None declared.

REFERENCES


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