Coagulation and inflammation in diabetes mellitus

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Renal tissue factor expression is increased in streptozotocin induced diabetic mice

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Abstract

**Background:** Tissue factor (TF) is a key initiator of the coagulation cascade. Recent evidence suggests that TF may play a role in renal fibrin formation and renal failure in experimental kidney disease. We hypothesized that hyperglycaemia will result in increased expression of TF, which might play a role in the pathogenesis of diabetic nephropathy.

**Methods:** Mice were injected with streptozotocin (STZ) (200 mg/kg) or with control buffer. Ten or 3 weeks after injection fibrin-, thrombin- and TF staining and TF activity were evaluated in the kidney. The effect of hyperglycaemia on TF expression and secretion by tubular epithelial cells was measured in vitro.

**Results:** Kidneys of STZ treated mice showed a marked increase in thrombin staining (3.0 ± 0.5 vs. 1.2 ± 0.11) (p=0.002) and an increase in TF clotting activity 10 weeks after STZ injection (33.9 ± 1.3 sec vs. 25.4 ± 1.0 sec) (p<0.0001). Increased glomerular fibrin deposition was present in 3 out of 6 diabetic mice. Tubular cells incubated with D-glucose (25 mmol/L) for 48 hr displayed increased cellular TF (P=0.05). However, soluble TF levels and TF activity in supernatant of cells incubated with D or L-glucose were not different.

**Conclusions:** This study shows an increased procoagulant state in the kidneys from STZ-injected mice and an increase in TF expression in HK2 tubular cells after incubation with high glucose concentration. These data suggest that hyperglycaemia induced procoagulant disturbances might play a role in the pathogenesis of diabetic nephropathy.
Introduction

Nephropathy is a common and serious complication of diabetes mellitus and is the major type of end-stage renal disease (ESRD) worldwide (1). Several large clinical trials have established that intensive diabetes therapy can reduce the risk of the development and progression of diabetic nephropathy (2; 3). However, its pathogenesis is still not well understood.

Fibrin/fibrinogen deposition in the glomeruli is a common feature of diabetic nephropathy (4; 5), suggesting that coagulation activation may be involved in the pathogenesis of diabetic renal disease. Furthermore, it has been frequently reported that diabetes mellitus is associated with changes in the hemostatic system (6-8). These changes are more pronounced in patients with diabetic nephropathy as compared to diabetic patients without renal complications (9-14) also suggesting that coagulation activation is associated with diabetic nephropathy.

Tissue factor (TF) is the primary cellular initiator of the coagulation cascade and can be expressed in a wide range of renal cell types, including tubular epithelial cells, parietal epithelial cells in Bowman’s space, endothelial cells and in the interstitium (15). Activation of the coagulation cascade via TF has been implicated in various renal diseases such as hydronephrosis (16), crescentic glomerulonephritis (17), lupus nephritis and endotoxin nephritis (18). Inhibition of TF activity with an anti-TF antibody can reduce glomerular fibrin deposition, reduce crescent formation and delay the development of renal failure in a model of experimental crescentic glomerulonephritis (19).

So far, there is only limited evidence that TF may play a role in diabetic kidney disease. Studies in obese, ob/ob and db/db mice with hyperinsulinemia and moderate hyperglycemia, due to a genetic leptin deficiency, or leptin receptor deficiency respectively, showed that TF mRNA is upregulated in the kidney of the mutant mice compared to their controls (20). Although there is evidence that hyperglycemic stress plays an important role in the renal injury of these obese mice (21), it is not clear whether the upregulation of TF in these models is due to hyperglycemia or to other (metabolic) factors. In addition, it is unclear which cell types express TF in the kidney and what influence hyperglycemia has on local activation of blood coagulation. The aim of our study was to investigate the influence of hyperglycemia on the expression of TF in the kidney in relation to other parameters of activated coagulation in a model of STZ induced diabetes.
mellitus in mice. To further explore the mechanisms of hyperglycemia related alterations of TF expression in tubulus cells we also analyzed TF synthesis by renal tubular epithelial cells after exposure to high glucose concentrations in vitro.

Materials and methods

Animals and Treatment

Female C57BL/6 mice were obtained from Charles River (Maastricht, The Netherlands). The study was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam, The Netherlands.

Diabetes mellitus was induced by a single intraperitoneal injection of streptozotocin (STZ) (200 mg/kg bw) in 0.05 M citrate buffer (pH 4) (n = 6). Blood glucose levels were measured with a Glucometer® Elite (Bayer Diagnostics, Mijdrecht, The Netherlands) in a drop of blood from the tail vein. If one STZ injection did not induce hyperglycemia (>10 mmol/L) after 4 days, the injection was repeated. Control mice were injected with citrate buffer alone.

Tissue harvesting and histological analysis

Three or 10 weeks after STZ or control injection mice were anaesthetized and killed by exsanguination. The right kidney was formalin-fixed, and the left kidney was directly put in liquid nitrogen and stored at -80°C. Formalin-fixed kidneys were routinely processed for paraffin embedding and 4 μm sections were serially cut and stained with H&E and PAS. For immunohistochemical analysis sections were incubated with 1.5% H₂O₂ in PBS for 20 minutes and then blocked with TENG-T (10mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% (vol/vol) Tween-20%, pH 8.0) for 30 minutes at room temperature. Thereafter sections were washed and incubated with primary antibodies against TF, thrombin and fibrin O/N at 4°C. Rabbit anti-mouse TF antibody and rabbit anti-mouse thrombin antibodies were developed in our laboratory by immunization of rabbits with a mixture of 500 μl Freund's Complete Adjuvant (Difco, Detroit, MI, USA) and murine TF peptide P5 (22; 23), or murine thrombin (Sigma, St.Louis, MO, USA). Final concentration of anti-thrombin antibody was 2.5 μg/ml and of anti-TF antibody 1.3 μg/ml. Rabbit anti-rat fibrin antibody was kindly provided by Dr. J. Emeis, TNO, The Netherlands and used in a 1:1000 dilution (24). After incubation with the primary antibodies tissue sections were washed and goat anti-rabbit (DAKO A/S, Glostrup, Denmark)
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was used as the secondary antibody in a 1:250 dilution for 1 hour at room temperature, and than washed with PBS. The sections were incubated with SABC complex (DAKO A/S) for one hour at room temperature, and washed. Enzyme activity was detected with AEC (Sigma) and 0.0075% H₂O₂ and counterstained with haematoxylin. Specificity controls included normal rabbit immunoglobulin in place of specific primary antibodies. For evaluation of immunostaining a semi quantitative score approach was chosen using the following criteria: 1: weak staining; 2: moderate staining; 3: strong staining.

In vitro studies
HK-2 cells (HPV 16-immortalized renal proximal tubular epithelial cell line obtained from normal adult human kidney) were purchased from ATCC (Rockville, MD, USA). Cells were grown to subconfluence in conditioned medium consisting of a 1:1 ratio of Dulbecco’s modified Eagle’s medium (DMEM) (ICN, Costa Mesa, CA) and Ham’s F12 (DMEM/F12) medium (Life Technologies, Paisley, UK) supplemented with 5% heat-inactivated FCS (Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM glutamine, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 20 ng/ml tri-iodothyronine, 5 ng/ml hydrocortisone, 5 ng/ml PGE1, 5 ng/ml epidermal growth factor, all obtained from Sigma (St. Louis, MO). For passage, confluent cells were harvested by trypsinization with 0.25% (w/v) trypsin-0.03% (w/v) ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), and plated again in conditioned DMEM/F12. For activation, cells were grown to confluence and were growth arrested in serum-free unconditioned medium for 24 hours, and all experiments were subsequently performed in unconditioned medium supplemented with 0.5% FCS. Cells at a concentration of 1 x 10⁶ cells / ml were incubated in 24-well plates with 25 mmol/l D-glucose for 24, 48 and 72 hours. Supernatants were collected and frozen in -20 °C. Osmolar control experiments were performed by the addition of 25 mmol/L L-glucose.

Tissue factor dependent procoagulant activation assay
Clotting assays of kidney homogenates from an independent experiment (n= 9 control mice; n=11 diabetic mice) and supernatant from the in vitro experiments were performed in a coagulometer (KC-10, Behring, Germany), which records the time taken for fibrin formation. Kidneys were homogenized in PBS (1 mg tissue: 10 ml PBS). To 100 μL of kidney homogenate, incubated in a coagulation
tube, 100 µL of commercially available mouse plasma (Sigma) was added. To 100
µL of supernatant 100 µL of human pool plasma from the blood of healthy donors,
who did not use aspirin or oral anticonception, was added. All components were
kept cold until use. The mixtures of either homogenates or supernatant with
plasma were incubated in the KC-10 for 2 minutes at 37.0 °C. Thereafter 100 ml 25
mM CaCl₂ was added and coagulation time was measured.

Western Blot analysis
For western blot analysis of TF in the different cell cultures, cells were lysed and
homogenized in 10 mM sodium phosphate buffer, 0.1 M e-aminocaproic acid, 5
mM trisodium EDTA, 10 aprotinin/ml, 10 U heparin/ml, and 2 mM pefabloc. The
homogenate was subjected to SDS-PAGE (10% running; 10% stacking gel) and
transferred to PVDF membrane (Immobilon-P: Millipore Corp., Bedford MA) by
electro blotting. TF was detected with a mouse α-human TF antibody (4509,
American Diagnostica, Stamfort, CT) and a chemiluminescence system (Lumi-
Light plus, Roche Diagnostics, Mannheim, Germany). TF expression after 24, 48
and 72 hours of incubation was expressed relative to 0 hour control incubation.
At least five different exposures (between 1 and 5 min) were obtained from each
membrane and analyzed with the GeneGnome software (Syngene, Cambridge,
UK).

Tissue factor antigen in supernatant
Soluble TF (sTF) was measured in supernatants of tubulus cells by ELISA (IMUBIND
TF, American Diagnostica). ELISA was performed as prescribed by the manufacturer.
Samples were prediluted 1: 5 in buffer. The absorbance of standards and samples
was read at 450 nm. The sTF concentration of the samples was calculated by
interpolation from the standard curve and corrected for the dilution factor using
the SOFTmax software from Molecular Devices Corporation (Sunnyvale, CA).

Statistics
Data are expressed as means ± SEM. Non-normal distributed data are expressed
as medians with 25-75 quartiles. For comparison between normal distributed
variables of interest the Student’s t test was used. The association between non-
normal distributed variables was assessed using the Mann-Whitney test. P values
< 0.05 are considered statistically significant.
Results

Induction of diabetes
Mice treated with STZ injection had significantly higher glucose levels both at 3 weeks after injection (20.1 ± 2.8 mmol/L N=6; 6.6 ± 0.50 mmol/L N=4; p=0.008) and at 10 weeks (27.6 ± 1.6 mmol/L N=6 vs. 7.9 ± 0.6 mmol/L N=6; p<0.0001) compared to control mice.

Morphology
After 3 weeks of hyperglycemia we could not observe any major differences between kidneys from STZ treated mice and control mice (data not shown). After 10 weeks of hyperglycemia however, we observed dilated tubules with flattened epithelial cells. Mesangial extracellular matrix was increased in glomeruli of STZ-mice (Fig. 1B). These changes were not found in kidneys from control mice (Fig. 1A).

Immunohistochemistry
No fibrin deposition was observed in control kidneys (Fig. 1C) and kidneys from mice 3 weeks after STZ injection (data not shown). Fibrin deposition was observed in glomeruli and in small vessels in 3 out of 6 kidneys from 10-week STZ mice (Fig. 1D). No fibrin was located in tubuli neither in experimental nor in control mice.

In all kidneys TF was expressed in the medulla located at the basal membrane of the tubuli (Fig. 2A and B). In the cortex TF was located in the cytoplasm (Fig. 2C and D). The majority of glomeruli stained negative for TF. If positivity was observed in glomeruli it was located in association with a passenger leukocyte or in parietal epithelial cells (Fig. 2E and F). Three weeks after diabetes induction we did not observe any differences in TF expression in the kidney compared to control mice.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Control (n=6)</th>
<th>Diabetes for 10 weeks (n=6)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Thrombin in cortical tubuli</td>
<td>1 (1-1)</td>
<td>3 (2-4)</td>
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<tr>
<td>Tissue Factor in cortical tubuli</td>
<td>2 (0.9-2.3)</td>
<td>2.5 (2-3)</td>
<td>0.1</td>
</tr>
<tr>
<td>Fibrin in glomeruli</td>
<td>1 (0-1.5)</td>
<td>1.5 (0-3)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 1. Data are presented as medians with 25th-75th percentiles. Semiquantitative score of staining with thrombin, TF and fibrin antibodies in kidneys from diabetic (10 weeks) and control mice. A significant increase was observed in thrombin staining in kidneys from mice 10 weeks after STZ injection.
Figure 1. A) PAS staining of a kidney from a control mouse. B) PAS staining of a kidney from a STZ induced diabetic mouse with tubular casts and widening of the tubuli. C) Absence of fibrin staining in glomeruli of control mouse. D) Fibrin deposition in glomeruli of STZ injected mouse. E) Weak thrombin staining in cortex from control mouse. F) Marked thrombin staining in cortical tubuli from STZ treated mouse. Positive staining was also located in the brush border of tubular cells (see insert). Magnification X 20, insert X 80.
Figure 2. TF staining in medulla located in basal membrane of tubuli in kidney from control (A) and STZ (B) treated mouse. Redistribution of TF was observed in the cortex of STZ-injected mice (D)* as compared to control mice (C). TF staining in parietal epithelial cells in control and diabetic mice (E and F)*. Magnification X 20 (A,B,C,D) and X 40 (E,F).
kidneys (data not shown). In the absence of a significant increase in TF expression, a more scattered pattern in the cortex was observed after 10 weeks of STZ-diabetes (Fig. 2D). Thrombin staining was very weak both in control kidneys (Fig. 1A) and in kidneys from mice 3 weeks after STZ injection (data not shown). Kidneys from mice that had been hyperglycemic for 10 weeks showed marked thrombin staining in the cytoplasm of tubular epithelial cells (Table 1), in the brush border and in perivascular capillaries (Fig. 1F).

Tissue factor dependent coagulation activation in kidney homogenates and in supernatant
To study the effect of diabetes mellitus on TF activity in the kidney a TF dependent coagulation activation assay was used. TF activity was similar in kidney homogenates from control mice and mice after three weeks of diabetes (control: $23.5 \pm 0.3$ sec N=4 vs. diabetes: $23.3 \pm 0.5$ sec N=6). TF activity was significantly increased in kidney homogenates from mice that had been diabetic for 10 weeks as compared to control mice (control: $33.9 \pm 1.3$ sec N=9 vs. diabetes: $25.4 \pm 1.0$ sec N=11) ($p<0.0001$) (Fig. 3).

Figure 3. Increased TF activity in kidneys from diabetic mice, evident from decreased coagulation times, compared to control mice using a TF dependent coagulation assay.

TF synthesis and secretion in vitro
The effect of hyperglycemia on TF protein levels was evaluated in vitro using western blot analysis of tubular epithelial cell lysates. After 24 hr there was no difference observed in TF protein in tubulus cells incubated with either D- or L-glucose (Fig. 4). However, after 48 hr incubation with D-glucose the relative cellular
level of TF measured by Western blotting did increase as compared to cells incubated with the osmotic control L-glucose (133.4 ± 12.9 vs. 200.6 ± 27.9 (p=0.05)) (Fig. 4). After 72 hr we still observed increased TF expression by 41 percent, but the increase was not significant.

In contrast, the level of soluble TF was not different between supernatants from cells that were incubated with either D- or L-glucose (Fig. 5). Accordingly, TF dependent coagulation in supernatant from tubulus cells incubated with D-glucose did not differ from that observed in osmotic controls at all different incubation times (Fig. 6).
Discussion

Nephropathy remains a frequent complication of diabetes mellitus and is regarded as an indicator of microvascular cell damage. Although a relation between hyperglycemia and the development of diabetic nephropathy has been established in patients with diabetes mellitus type 1 and 2, the pathogenesis is still not well understood.

Fibrin/fibrinogen deposition is a common feature in the glomeruli of diabetic kidneys (4; 5), thought to contribute to thrombus formation in the renal microvasculature which may accelerate loss of renal function. The presence of fibrin, the final product of the coagulation cascade, in glomeruli suggests the participation of the coagulation system in the pathogenesis of diabetic nephropathy (4; 5). To some extent this process may be influenced by the hypercoagulable state in blood from diabetic patients, which is even more pronounced in patients with nephropathy (9-14). In analogy with other renal diseases we speculated that local activation of the blood coagulation system, particularly the generation of TF and specific proteases such as thrombin that may induce biological effects unrelated to fibrin formation, would contribute to organ damage.

In the present study we utilized a well characterized model of diabetic nephropathy, i.e. STZ induced hyperglycemia in mice. At 10 weeks after STZ challenge and upon stably induced hyperglycemia, evidence of nephropathy was clearly present in all treated mice. We demonstrated an increased activity of TF in the kidneys of STZ treated mice associated with an altered pattern of cellular expression confined to tubulus cells. A possible functional association between tubular TF activity and activated coagulation is suggested by the increase in local thrombin staining. However, fibrin was present more abundantly in the glomeruli of STZ treated mice, but was not found in tubuli, while, in contrast glomeruli where fibrin localized did not contain increased staining of TF. The apparent dissociation in localization of TF and thrombin vis à vis fibrin in the kidney cannot be interpreted with certainty. This spatial dissociation differs from observations in other models of renal disease. i.e. glomerulonephritis (19). In the latter study, TF and fibrin colocalized in the glomeruli and inhibiting TF with anti-TF antibody reduced glomerular fibrin deposition suggesting a functional relation between TF and the clotting endproduct fibrin (19). In our model, the increased TF activation in tubular cells might have led to increased release of soluble forms of TF that would trigger coagulation and lead
to fibrin formation at distant sites such as glomeruli. More likely, local TF expression indeed triggers thrombin generation which binds to cell-surface proteinase activated receptors (PAR’s) to induce cell signaling mechanisms (25; 26), while fibrin deposition in glomeruli has another (e.g. blood born) origin. Although STZ induced diabetes mellitus is characterized by the typical features of diabetes type 1, such as loss of insulin production, hyperglycemia, glucosuria and weight loss, it is not clear whether the renal changes in STZ induced diabetic mice were due to hyperglycemia or in part be due to secondary mediators also present in other renal diseases, including pro-inflammatory cytokines that may influence the level of TF expression as part of the innate immune response to injury. Furthermore, upregulation of TF is not a specific feature of STZ induced nephropathy, but has also been reported in various other models of experimental renal diseases. Finally, the apparent increased activity of TF may be a consequence of conformational changes in TF molecules or increased shedding of active forms of TF from the cell surface. To clarify whether hyperglycemia induces upregulation of TF expression in tubulus cells we incubated tubular epithelial cells in vitro with a high concentration of glucose and assessed the synthesis and secretion of TF after different exposure times. We observed that upon prolonged incubation with high glucose TF expression was higher than in control cells. This made us conclude that hyperglycemia itself induces enhanced TF expression. It is tempting to speculate that tubulus cell-related TF acts as a receptor for factor VIIa leading to cell signaling cascades related to inflammation or angiogenesis (27). While increased production of TF by tubulus cells was demonstrated, increased shedding in supernatant was not shown, suggesting that increased release of soluble forms of TF is not involved in distant (glomeruli) fibrin formation. The origin of glomeruli related fibrin remains unknown, but may be due to enhanced deposition of intravascularly generated fibrin molecules, secondary to microvascular “damage”.

The altered expression of TF in the in vitro experiment was seen after 48 hours of hyperglycemia and not after 24 hours, indicating that the deteriorating effect of hyperglycemia on tubular cells requires prolonged glucose exposure. Although extrapolating in vitro to in vivo data may not be entirely appropriate, the increased staining in thrombin and increased activity of TF in the in vivo model of diabetic nephropathy was significant only after 10 weeks of diabetes duration but not
after 3 weeks. The signs of renal injury in the STZ model including widened tubuli and tubular casts were also only seen after 10 weeks, but not after 3 weeks. This is in line with earlier observations in the model of STZ diabetes; showing that the severity of renal injury, i.e. proteinuria, increased thickness of GBM, increased kidney size and dilatation of tubuli, is progressive over time in diabetes (28). These observations underline that hyperglycemia has a protracted deteriorating effect on renal function and is supported by the fact that patients with diabetes generally develop renal problems after several years of diabetes (2).

In conclusion, the present data show that experimental diabetic nephropathy is associated with locally enhanced expression of proteins involved in activated blood coagulation. The localization and activity of the procoagulant proteins TF and thrombin confined to tubular cells suggest a role in intracellular signaling in response to chronic hyperglycemic injury. Fibrin formation, the conventional endpoint of the coagulation cascade, is visible in some diabetic kidneys but is associated with glomeruli and may be to a certain extent a marker of microvascular damage due to its localization distant from the other coagulation markers. Together, the data suggest a role for renal blood coagulation proteins in the local response to hyperglycemic injury in diabetic nephropathy, but specific interventions with anticoagulant agents need to prove the relevance of this pathophysiological scenario.

Acknowledgments

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Reference list

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