Identification of a Unique Glomerular Factor X Activator in Murine Lupus Nephritis

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Abstract. The role of glomerular procoagulant activity (PCA) was studied in mice (MRL/lpr, NZBxWF1, and BXSB) that are known to develop lupus nephritis. In young mice (6 to 8 wk) without renal disease, there was no increase in spontaneous glomerular PCA. In contrast, older (5 to 8 mo) autoimmune mice had significant augmentation in glomerular PCA, coinciding with the histologic appearance of severe glomerulonephritis and renal fibrin deposition. The PCA was characterized as a serine protease that directly activated factor X. This factor X activator is not tissue factor because (1) expression of PCA was not dependent on factor VII; (2) a monoclonal antibody against the factor X activator inhibited glomerular PCA, but not tissue factor; (3) the molecular weight (66 kD) of the activator was different from that of tissue factor; and (4) concanavalin A inhibited tissue factor but not glomerular PCA. Immunohistochemical studies localized the factor X activator to the glomerular mesangium and capillary wall of 4- to 6-mo-old diseased MRL/lpr mice. Immunogold-labeled antibody bound to the dense deposits, macrophages, and endothelial cells of diseased glomeruli. These studies define the role of a unique glomerular factor X activator in murine lupus nephritis.

Fibrin deposition is associated with many forms of animal and human glomerulonephritis (GN) and has been detected in glomeruli with both endocapillary and extracapillary proliferative lesions (1), where it may contribute to injury. In support of this concept, defibrination reduces glomerular damage in animals and humans (2–4). The mechanism leading to fibrin deposition is unclear but may be secondary to local or systemic activation of coagulation. A potential trigger for fibrinogenesis is the increased glomerular procoagulant activity (PCA), which has been found in a number of models of GN including nephrotoxic nephritis, acute serum sickness, mercuric chloride-induced GN, and in human proliferative GN (5–7). The cells responsible for enhanced glomerular PCA may be structural glomerular cells such as endothelial, mesangial, and epithelial cells (8–11), or blood-borne cells such as monocyte-macrophages that infiltrate glomeruli. A number of cellular procoagulants have been described previously, including tissue factor, and activators of factor X and prothrombin (12–15).

Our previous studies have shown that circulating monocytes from patients with active lupus nephritis have increased monocyte-macrophage PCA (16). It also was shown that in the male BXSB murine model of lupus nephritis, plasma-stimulated lymphocytes induce splenic macrophage PCA, which occurs in concert with the evolution of the glomerular disease (17). Ancrod, a defibrinating agent, reduced macrophage PCA and glomerular fibrin deposition, and improved both renal function and survival (4).

To gain a greater understanding of the relevance of local activation of coagulation in the renal disease associated with lupus, the current studies were designed to assess, characterize, and localize glomerular PCA before and after the development of the disease. Studies were also undertaken to assess the correlation between the expression of this unique glomerular PCA and the severity of the glomerular disease with the deposition of fibrin in the glomeruli.

Materials and Methods

Mice

Male BXSB, female MRL/lpr, and male Balb/cJ mice, 6 to 8 wk and 4 to 5 mo of age, female NZBxWF1 mice, 6 to 8 wk and 8 to 9 mo of age, and A/J and CAF1 mice, 6 to 8 wk of age, were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the Department of Laboratory and Animal Services at the University of Toronto.

Virus

Murine hepatitis virus strain 3 (MHV-3) was plaque-purified on monolayers of DBT cells and grown to titers of $1.0 \times 10^7$ plaque-forming units/ml in 17 CL1 cells. Viral titers were determined on monolayers of L2 cells in a standard plaque assay (18).
Cells
Peritoneal macrophages were harvested from Balb/cJ mice 4 d after injection with 2 mL of 3% thioglycollate (DIFCO Laboratories, Detroit, MI), as described previously (18). After being washed, cells were resuspended at 2 × 10^6/mL in RPMI 1640 (ICN Biomedicals, Costa Mesa, CA) supplemented with 2 mM glutamine (Sigma Chemical Co., St. Louis, MO) and 2% heat-inactivated fetal calf serum (Life Technologies, Burlington, Ontario, Canada). Viability exceeded 95% by trypan blue exclusion. Macrophages were stimulated with either 10 µg of lipopolysaccharide (LPS) (Sigma) or with MHV-3 at multiplicity of infection of 2.5. Eight hours later, macrophages were recovered after two washes with fresh RPMI 1640. Cells were frozen and then assayed for PCA (see below).

Glomerular Isolation
Mice were sacrificed by exsanguination. Both kidneys were removed aseptically and placed in cold 0.1 M phosphate-buffered saline (PBS), pH 7.2. After removal of the capsule, each kidney was bisected and the bulk of the medulla was removed and discarded. The remaining tissue (cortex) was minced and passed through a stainless steel sieve fitted with a 230-µm sieve (EC Apparatus Corp., VWR Scientific Canada, Ltd., London, Ontario, Canada). The sieve was washed and the tissue was collected in PBS and centrifuged at 2000 rpm for 5 min at 4°C. The pellet was resuspended in a small volume of PBS and passed through a 10 cc syringe fitted with a 23-gauge needle twice. The solution was centrifuged again at 2000 rpm for 5 min and resuspended in a small volume of PBS. The mixture was passed through a nylon mesh with 105-µm pore size (Thompson Company Ltd., Scarborough, Ontario, Canada) and collected on a nylon mesh of 53-µm pore size. All of the material retained on the 53-µm pore size mesh was collected, and homogeneity of the suspension was assessed microscopically and found to be at least 90% pure for glomeruli. After counting, 3000 glomeruli were aliquoted into each tube. Glomeruli were kept frozen at −70°C until used. To determine the total PCA, frozen glomeruli were thawed and then ground with a Micro tissue grinder until no intact glomeruli were evident by microscopy. Preliminary studies showed that PCA was predominantly expressed by the glomeruli and not the tubules or interstitium (data not shown).

PCA
Samples were assayed for PCA based on their ability to accelerate the spontaneous clotting time of recalcified platelet-poor normal human plasma as described previously (17). Some studies were also performed using platelet-poor normal rat plasma. Rat blood was collected from the aorta aseptically, and plasma was prepared in a manner similar to human plasma. To determine PCA, 300 glomeruli/assay or 20,000 macrophages/assay were used. Milliunits of PCA were assigned by reference to a standard curve generated with serial dilutions of rabbit brain thromboplastin (Sigma). The medium and reagents were without activity. Additional PCA assays were performed with human plasmas deficient in coagulation factors II, V, VII, VIII, IX, X, XI, and XII (Helena Laboratories, Beaumont, TX) to determine the nature (factor dependence) of the glomerular PCA.

Endotoxin Contamination
All media and buffers were assayed for endotoxin contamination using a standard limulus assay (Escherichia Toxate; Sigma) and contained <0.1 ng/ml endotoxin, the lower limit of detection of the assay.

Glomerular Lysate-Mediated Cleavage of Factor X and II
Factor X and prothrombin (Sigma) were radiolabeled with 125I enzymatically using immobilized laccoperoxidase and glucose oxidase (Enzymebeads; Bio Rad Laboratories, Mississauga, Ontario, Canada) according to the product information. A lysate from 300 glomeruli was incubated at 37°C with 0.06 ng of factor X with 125I-labeled factor X diluted in 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.8, containing 20 µL of 25 mM CaCl₂. Alternatively, the glomerular lysate was incubated with 0.6 or 72 ng of factor X in the presence of 125I prothrombin and 20 µL of 25 mM CaCl₂. The incubation was terminated after 60 min by adding 10% sodium dodecyl sulfate and 10% ethylene diaminetetra-acetic acid, both to a final concentration of 1%. Thereafter, samples were loaded on to 10% sodium dodecyl sulfate-polyacrylamide gels and electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (19). Autoradiography was performed after exhaustive washing, and the pattern of factor X or prothrombin cleavage was compared to that produced by incubation with 0.02 or 0.6 ng Russell’s viper venom (RVV) (Sigma).

Factor X Chromogenic Cleavage Assay
To assess the ability of diseased glomeruli to directly activate factor X, the Chromozyme X⁰ kit (Boehringer Mannheim Biochemica, Laval, Quebec, Canada) was used as in the protocol supplied with the kit. The reaction uses Chromozyme X⁰ (Methoxy carbonyl-Norleucyl-Gly-Arg-4-Na), a synthetic substrate that is cleaved by activated factor X (factor Xa). Cleavage of the substrate 4-Nitranilin, which can be detected photometrically as an increase in absorbance at 405 nm. The reaction mixture included the glomerular extract (600 glomeruli), 16 mM CaCl₂, and 0.6 ng of human factor X (Boehringer Mannheim Biochemica, Laval, Quebec, Canada). The same mixture without glomeruli was used as a control. The procedure involved adding the reaction mixture and sample to individual wells of 96-well enzyme-linked immunosorbent assay plates (ICN Flow Laboratories, Mississauga, Ontario, Canada), mixing, and incubating for 3 min at room temperature. The substrate solution was added and absorbance at 405 nm was assessed over time using a Titer Tek Mutliiskan MCC/340 (Flow Laboratories).

Inhibition of PCA
To further characterize glomerular PCA, glomerular extracts were incubated with concanavalin A (ConA) at 10 µg/ml, aprotinin at 50 µg/ml, phenylmethylsulfonyl fluoride (PMSF) at 500 µg/ml, trans-epoxysuccinyl-L-leucylamido (4-guanidino)-butane (E-64) at 0.5 mM (all inhibitors were obtained from Sigma), or buffer for 30 min at 37°C, and then assayed for PCA as described above.

Production of Monoclonal Antibody against Glomerular PCA
MRL/lpr mouse glomeruli were isolated from 5-mo-old mice (those with severe GN and expressing high glomerular PCA) as described above. Glomeruli were mixed with 100 µl of killed Bordetella pertussis (2 × 10⁹ cells) and injected into the peritoneal cavity of A/J mice. Fourteen and twenty-one days later, animals were boosted intraperitoneally with glomeruli expressing high PCA. On day 35, the animals were boosted one additional time with PCA-positive glomeruli, and 24 h later spleens were removed. Fusion of spleen cells to the SP2/0 myeloma cell line and isolation of hybridomas were performed as described (20,21). Screening and selection of the hybridomas were done by inhibition of glomerular PCA using the one-stage clotting assay described below. Each hybridoma was sub-
Inhibition of PCA by Monoclonal Antibody (H4)

The ability of H4 to inhibit glomerular PCA was determined by mixing equal volumes of glomeruli from diseased MRL/lpr mice (300 glomeruli) and H4 purified from ascites (4 μg) for 30 min at room temperature. PCA was measured by adding 100 μl of the incubation mixture to 100 μl of normal citrated human plasma or 100 μl of normal citrated rat plasma and 100 μl of 25 mM CaCl₂ and determining the clotting time. The ability of normal mouse IgM to inhibit glomerular PCA was assessed as described for H4. The effect of H4 on LPS-stimulated macrophages was also studied. All assays were performed four times in duplicate.

Western Blotting with H4

Proteins were extracted from 3000 glomeruli, 1.0 × 10⁶ MHV-3-stimulated Balb/cJ macrophages, or 1.0 × 10⁶ LPS-stimulated Balb/cJ macrophages with a buffer containing Tris-HCl (50 mmol/L, pH 7.5), NaCl (150 mmol/L), Nonidet P-40 (1% vol/vol), and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mmol/L PMSF). Samples were subjected to 10% SDS-PAGE according to the method of Laemmli (19), followed by transfer to nitrocellulose by the method of Burnette (22). The membrane was then blocked with 4% nonfat milk in buffer containing 0.1% Tween 20 in Tris-buffered saline for 5 min each, silver-enhanced for 5 min with the HQ SILVER® film (Kodak). Samples were subjected to enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL). A control blot was performed exactly as described above, but without H4 to exclude the possibility of nonspecific binding due to the second antibody.

Morphology

Four- to six-month-old MRL/lpr mice were sacrificed when they developed heavy proteinuria detected using Albustix® (Bayer Corp., Leverkusen, Germany). Kidney cortex was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin and Picro-Mallory stain. A light microscopic scoring scheme was devised to facilitate quantification of the sequence of glomerular events. Scoring was as follows: −, normal glomerulus; +, predominantly mesangial proliferation; ++, moderate to severely affected glomeruli (endocapillary proliferation with necrosis and/or crescents). The presence or absence of fibrin (from Picro-Mallory staining) in glomeruli was noted.

Immunofluorescence Microscopy

Old and young MRL/lpr mice were sacrificed, and pieces of kidney cortex, liver, spleen, heart, and lung were embedded in OCT compound (Miles, Elkhart, IN) and snap-frozen in liquid nitrogen. Four-micrometer sections were cut, fixed in methanol for 5 min, and blocked with 5% horse serum in PBS for 2 h. The sections were then stained with H4 or normal mouse IgM for 2 h with H4 and normal mouse IgM that were labeled with FITC using a Calbiochem FITC labeling kit (La Jolla, CA). After five 10-min washes in PBS containing 0.05% Tween 20, sections were mounted in 90% glycerol in PBS and viewed on a Leitz phase/epifluorescence microscope equipped with a ×40 Fluotar objective. Photographs were taken on Ektachrome film (Kodak).

Immunogold Staining

To localize the expression of glomerular factor X activator at the ultrastructural level, H4 was labeled with NANOGOLD™ (Nanoprobes, Inc., Stony Brook, NY) according to the product information. For this purpose, 3-mm pieces of kidney cortex from five 4- to 6-mo-old diseased MRL/lpr mice and four 6- to 8-wk-old normal MRL/lpr mice were fixed by immersion in 4% paraformaldehyde for 4 h and then rinsed in 0.1 M phosphate buffer. The tissue was then stored in 0.02 M sodium azide in 0.1 M phosphate buffer until it was embedded in Lowicryl KM4 (JBFM Services, Dorval, Montreal, Quebec, Canada). Ultrathin sections were cut and mounted on carbon-coated nickel grids. Sections were blocked with 0.15% glycine in PBS buffer at pH 7.4 three times and then with 0.5% bovine serum albumin in PBS three times, 5 min each. Grids were then incubated with NANOGOLD™-conjugated H4 diluted 1:20 in PBS-bovine serum albumin for 1 h at room temperature and rinsed in PBS four times, 5 min each. They were then post-fixed with 1% glutaraldehyde in PBS at room temperature for 3 min, rinsed in deionized water four times for 5 min each, silver-enhanced for 5 min with the HQ SILVER® silver enhancement system (Nanoprobes), and rinsed three times in deionized water. Finally, sections were contrasted with uranyl acetate and lead citrate before examination under the electron microscope.

Figure 1. Effect of age on murine glomerular procoagulant activity (PCA). Spontaneous PCA of glomerular lysates (3000 glomeruli) from young (6 to 8 wk) and old (4- to 6-mo-old Balb/cJ, BXSB, and MRL/lpr, and 8- to 9-mo-old NZBxWF1) mice of four different strains was measured in a one-stage clotting assay. In glomeruli from Balb/cJ mice, there was no significant increase in PCA with advancing age. In contrast, PCA increased with age in the three lupus-prone strains of mice. Results represent the mean ± SD of four experiments done in triplicate. *P < 0.05.
Results

Glomerular Procoagulant Activity

Data comparing spontaneous PCA of glomerular lysates from young and old mice of several different strains are shown in Figure 1. In glomeruli from Balb/cJ mice, there was no significant increase in PCA with age. In contrast, PCA increased with age in glomeruli from the three mouse strains that develop lupus nephritis. Further characterization and other studies of glomerular PCA were performed on the MRL/lpr strain.

Characterization of Glomerular PCA

The factor dependence of glomerular PCA and tissue factor was compared using glomerular lysates from 5-mo-old MRL/lpr mice, LPS-stimulated peritoneal macrophages, and human plasma congenitally deficient in the various clotting factors. The data shown in Table 1 indicate that factors II, V, and X were required for glomerular PCA, whereas factors VII, VIII, IX, XI, and XII were not needed. In contrast, tissue factor mediated PCA required the presence of factors II, V, VII, and X for its activity.

Activation of Factors X and II

The nature of glomerular PCA was examined further by assessment of the ability of glomerular lysates from 5-mo-old MRL/lpr mice to cleave radiolabeled factors X and prothrombin. As indicated in Figure 2, the lysate cleaved factor X in a manner analogous to RVV, which was used as a positive control for factor X cleavage. The glomerular lysate cleaved prothrombin in the presence of factor X. In Figure 3, the lysate was able to cleave factor II in the presence of factor X, but not in its absence. Cleavage of factor X by glomerular lysate was calcium-dependent (data not shown).

Cleavage of Factor X Chromogenic Substrate

The ability of the glomerular lysate to directly cleave factor X was assessed using the factor X chromogenic substrate. These data are shown in Figure 4. Cleavage of factor X was seen as early as 30 min, increasing over 1 h with maximum activity at 180 min. The time course for cleavage of the chromogenic substrate was similar to that of $^{125}$I factor X, although minor differences may reflect sensitivity of the assays and the smaller number of cells used in the assay (15). When larger numbers of glomeruli were used ($>10^3$), the time course was accelerated with $>80\%$ of substrate cleared within 60 min (data not shown).

Table 1. Effect of factor-deficient plasmas on glomerular PCA in MRL/lpr mice and tissue factor PCA

<table>
<thead>
<tr>
<th>Plasma Deficient in Factor</th>
<th>PCA (mU/3000 glomeruli)</th>
<th>PCA (mU/10⁶ LPS macrophages)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2192 ± 408</td>
<td>1441 ± 341</td>
</tr>
<tr>
<td>II</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>V</td>
<td>390 ± 48</td>
<td>&lt;10</td>
</tr>
<tr>
<td>VII</td>
<td>2112 ± 522</td>
<td>&lt;10</td>
</tr>
<tr>
<td>VIII</td>
<td>1338 ± 0</td>
<td>1097 ± 476</td>
</tr>
<tr>
<td>IX</td>
<td>2176 ± 136</td>
<td>ND</td>
</tr>
<tr>
<td>X</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>XI</td>
<td>2176 ± 136</td>
<td>ND</td>
</tr>
<tr>
<td>XII</td>
<td>1750 ± 218</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a* PCA, procoagulant activity; LPS, lipopolysaccharide; ND, not determined.

Effect of Inhibitors

The effect of inhibitors on glomerular factor X activator and tissue factor activity is presented in Figure 5. There was no significant effect of E-64 on glomerular factor X activator or tissue factor-mediated PCA. ConA did not significantly inhibit glomerular factor X activator at 10 μg/ml, a concentration that inhibited tissue factor PCA by $>90\%$. Aprotinin inhibited glomerular factor X activator by approximately 70% and tissue factor...
PCA by 50%, whereas PMSF inhibited glomerular factor X activator and tissue factor PCA by 80 and 75%, respectively.

Effect of Monoclonal Antibody to Glomerular PCA (H4) on Glomerular Factor X Activator and Tissue Factor

The effect of H4 on PCA induction by glomeruli and LPS is compared in Figure 6 for both human (Panel A) and rat (Panel B) plasma. It can be seen that PCA using rat plasma is substantially higher than with human plasma. However, in both cases, the results are qualitatively similar in that H4 inhibited glomerular PCA by approximately 75%, but did not significantly inhibit tissue factor (<20%, P = NS).

Western Blot Analysis

Western blot analysis using H4 monoclonal antibody was performed to identify the molecular weight of the factor X activator (Figure 7). H4 bound to a protein with a molecular weight of 66 kD in glomeruli expressing high PCA from...
5-mo-old MRL/lpr mice. The band was absent in glomeruli expressing low PCA from 8-wk-old MRL/lpr mice. There was no binding to macrophages expressing tissue factor (LPS-stimulated) or direct prothrombinase (MHV-3-stimulated), and there was no binding in the control blot using the second antibody alone (data not shown).

Morphology and Immunofluorescence Microscopy

Table 2 summarizes the light microscopic observations and the immunofluorescence results. Figure 8 illustrates the results obtained when direct immunofluorescence using H4 was performed on old and young MRL/lpr mouse kidney sections. Staining was seen in glomeruli of 5-mo-old MRL/lpr mouse with severe GN, mainly in the mesangium and capillary walls (Figure 8B). Immunofluorescence was negative on 8-wk-old MRL/lpr mouse kidney (Figure 8A) and on young and old MRL/lpr mouse liver, spleen, heart, and lung (data not shown).

Binding of fluorescence-labeled H4 was seen in 10 of 13 mice with moderate to severe glomerular lesions characterized by diffuse and focal endocapillary proliferation with necrosis and/or crescents, and in two of seven mice with mild glomerular lesions characterized by mesangial proliferation. In con-

![Figure 7. Western blot analysis using H4. Diseased MRL/lpr mouse glomeruli with PCA of 6000 mU/3000 glomeruli (lane 1), young MRL/lpr mouse glomeruli with PCA of 400 mU/3000 glomeruli (lane 2), LPS-stimulated macrophages with PCA of 3500 mU/10⁶ cells (lane 3), or murine hepatitis virus strain 3 (MHV-3)-3-stimulated macrophages with PCA of 4000 mU/10⁶ cells (lane 4) were resolved by electrophoresis, transferred to nitrocellulose, and reacted with H4.](image)

![Figure 8. Immunohistochemistry. Normal and diseased MRL/lpr mouse kidney sections were stained with FITC-labeled H4 against factor X activator. (A) Young, normal MRL/lpr kidney stained with H4, which was negative. (B) Diseased MRL/lpr kidney stained with H4 with evident antibody binding primarily to both glomerular mesangium and endothelium. Magnification, ×100.](image)

<table>
<thead>
<tr>
<th>Glomerular Morphology</th>
<th>No. of Mice</th>
<th>No. of Mice with Fibrin</th>
<th>No. of Mice with Factor X Activator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (−)</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mesangial proliferation (+)</td>
<td>7</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Focal or diffuse endocapillary</td>
<td>13</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>proliferation (+++)</td>
<td></td>
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</table>
Ultrastructural Localization of Factor X Activator

The immunogold staining results are presented in Figures 9 and 10. There was no glomerular binding of immunogold-labeled H4 in any of the young, healthy mice (Figure 9A). Four- to six-month-old MRL/lpr mice with severe glomerular lesions characterized by endocapillary proliferation with necrosis and crescent formation had intense staining as indicated by numerous silver grains in the dense deposits found in the subendothelial zone of the basement membrane (Figure 9B). It was also found in subendothelial deposits, which contained fibrin-like structures (Figure 10A). Phagosomes contained within mesangial macrophages show extensive staining (Figure 10B). Deposition of H4 was also found in phagosomes and endoplasmic reticulum within glomerular macrophages and/or endothelial-like cells (not shown).

Discussion

The present studies were undertaken to quantify, characterize, and assess the role of glomerular PCA in mice that develop lupus nephritis. Glomerular PCA increased with age in the three murine lupus models. In contrast, there was no age-associated increase of glomerular PCA in Balb/cJ mice, a strain that does not develop GN. The increased glomerular PCA in the MRL/lpr mice had the characteristics of a factor X activator based on the following: (1) only coagulation factors X, V, and prothrombin were needed for full expression of this activity; (2) the glomerular lysate from MRL/lpr mice directly cleaved $^{125}$I-factor X and factor X chromogenic substrate; and (3) no activation of prothrombin occurred in the absence of factor X, thereby excluding the presence of a direct prothrombinase. We believe that the small amount of glomerular PCA (20%) seen with factor V-deficient plasma likely relates to small amounts of factor V present in the glomeruli.
The possibility of glomerular factor X activator being tissue factor was excluded by several observations. First, glomerular PCA did not require factor VII for its expression. Although it may be argued that there could be elevated levels of factor VII in the glomeruli due to the synthesis of factor VII by local cells, as has been described for macrophages (23), other observations provide evidence to the contrary. The molecular weight of the factor X activator is approximately 66 kD, whereas the molecular weight of tissue factor is 31 kD for the nonglycosylated form and 46 kD for the glycosylated form. H4, a monoclonal antibody that inhibited glomerular factor X activator, did not react with tissue factor on immunoblot and failed to inhibit macrophage PCA induced by LPS, which is known to be tissue factor. This differential inhibition occurred using both human and rat plasma. The experiments were performed with rat plasma to ensure that murine tissue factor was completely expressed. Finally, ConA, at doses that blocked tissue factor, had no effect on glomerular factor X activator. The molecular weights of factor VIIa (32 kD) (24) and factor IXa (58 kD) (25) are different from the glomerular factor X activator, eliminating these other possibilities.

Our previous studies on splenic and peritoneal macrophages in BXSB mice had suggested that the induced PCA was a prothrombinase (17). These current studies, showing a factor X activator in glomeruli from mice, are at odds with our previous studies. However, the mouse strains and cell type in the two studies differ. In addition, we believe that the use of a specific antibody against the glomerular PCA (which did not react with or inhibit murine prothrombinase; data not shown) and the molecular weight of the factor X activator (which is different from murine prothrombinase) support our hypothesis that the glomerular factor X activator is unique.

Furthermore, Edgington and others have now unequivocally shown that LPS induces tissue factor in rodents (26). Thus, our previous observations suggesting that LPS-induced PCA was a prothrombinase are not borne out (27). It is possible that the splenic macrophages in our earlier studies may have been contaminated with small numbers of lymphocytes. LPS could have induced interferon-γ, which has been demonstrated to induce prothrombinase (28).

Immunofluorescence studies using H4 revealed that factor X activator expression was detected in close proximity to fibrin-like structures in the subendothelial deposits. Electron microscopic studies with immunogold-labeled H4 localized the factor X activator to dense deposits in the subendothelial zones, mesangial macrophages, and the endoplasmic reticulum of endothelial cells. Kanno and colleagues have demonstrated that macrophages can phagocytose material deposited in the subendothelial and mesangial regions (29). Thus, it is possible that the factor X activator detected in the phagosomes of glomerular macrophages could be derived from deposits phagocytosed by the macrophages.

In the present study, glomerular factor X activator was present only in areas of active glomerular disease. Thus, it is possible that inflammatory cells at these sites release cytokines that induce the expression of glomerular factor X activator. Wharram et al. have suggested that monocyte-to-endothelial cell interaction modulates PCA in the presence of immune complexes (30).

A number of factor X activators have been described previously. Activation of factor X by a serine protease with the properties of a tissue factor–factor VIIa complex has been demonstrated in normal and malignant tissue from human colon and breast (31,32). Shands has described a factor X activator in mouse peritoneal exudate macrophages that also appears to be a complex of membrane-bound tissue factor and factor VII-like substance, produced by the macrophages themselves (33). Gordon and Cross have identified a cysteine protease that activated factor X, in extracts of human and animal tumors and in cultured malignant cells (34). More recently, the production of a factor X activator by a methylocholanthrene-induced rat fibrosarcoma has also been described (35). This activity was similar to the glomerular factor X activator in terms of its relative resistance to ConA and E-64 and sensitivity to inhibition by aprotinin.

The exact role of glomerular PCA in the pathogenesis of glomerular disease is not clear. A number of investigators have documented glomerular production of tissue factor in other models of GN (23,36–38). However, this is the first report of a glomerular factor X activator. The factor X activator could cause tissue damage by inducing fibrin formation within the microcirculation. Fibrin deposits reduce macrophage mobility, are cytotoxic to mesangial cells, and attract circulating inflammatory cells (39–41). Recently, studies have suggested that proteases of the coagulation cascade may transduce intracellular signals to modulate immunologically induced inflammation (42). Activation of the thrombin receptor on leukocytes has been shown to increase cytosolic free Ca, stimulate mitogen-activated protein kinase activation, tyrosine kinase phosphorylation, and the release of hemostatic and inflammatory cytokines (43,44). Altieri and colleagues have now shown that injection of factor Xa into rat paw induced inflammation, which was not attributable to thrombin (45). They have demonstrated that the vascular factor Xa receptor, effector cell protease receptor-1, can trigger leukocyte activation and smooth muscle cell proliferation in addition to endothelial cell thrombin generation (42,46,47). Factor Xa also enhances release of endothelial cell mitogens such as platelet-derived growth factor-like molecules (48). Thus, glomerular factor X activation could contribute directly through a nonclassical coagulation pathway to the glomerular inflammation in murine lupus nephritis.

In renal biopsies from patients with human lupus nephritis, subendothelial immune globulin and fibrin are associated with endothelial injury. As well, gaps in the glomerular basement membrane have been associated with intracapillary fibrin deposition and fibrinogen-related antigen in Bowman’s space. Glomerular thrombi are common, having been found in 92 of 170 renal biopsy specimens in lupus patients with diffuse proliferative GN (49). Glomerular thrombi in the initial renal biopsy specimen were predictive of increased glomerular sclerosis in a subsequent renal biopsy and were found to be more predictive than other histologic findings including crescents, necrosis, and subepithelial deposits. Ancrod, a defibrinating
agent, improved both histologic findings and renal function in Pollak’s human study (49). Thus, glomerular thrombi and fibrin play an important role in the genesis of severe human glomerular injury and act as a precursor of chronic irreversible damage. The information presented here and our previous data on increased monocyte PCA in patients with human diffuse proliferative lupus nephritis (16) suggest that glomerular factor X activator could play a significant pathogenic role in human lupus nephritis.

Acknowledgments

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