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CHARACTERIZATION AND LOCALIZATION OF GLOMERULAR FACTOR X ACTIVATOR IN MURINE LUPUS NEPHRITIS

by

Subodini Perampalam

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Cellular and Molecular Pathology,
University of Toronto

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ABSTRACT

CHARACTERIZATION AND LOCALIZATION OF GLOMERULAR FACTOR X ACTIVATOR IN MURINE LUPUS NEPHRITIS

Master of Science, 1997
Subodini Perampalam

Cellular and Molecular Pathology, University of Toronto

Glomerular fibrin deposition and induction of cellular procoagulant activity (PCA) are associated with both human and murine glomerulonephritis (GN). In order to examine the relationship between murine GN and glomerular PCA, we compared glomerular PCA in normal mice (Balb/cJ) and in three strains of lupus mice (MRL/lpr female, NZBxWF1 female, and BXSB male) that develop immune complex mediated GN spontaneously. In young mice (age 6-8 weeks), at a time when there was no histologic evidence of renal disease, there was no difference in the spontaneous glomerular PCA between the normal and the lupus prone mice. However, older (5 - 8 months) autoimmune mice, but not Balb/cJ mice showed a significant augmentation in glomerular PCA, which coincided well with the histologic appearance of severe GN. Glomerular PCA was characterized as a direct activator of factor X because (a) expression of its activity required the presence of factors X, V and II (prothrombin), and (b) incubation of glomerular lysates with $^{125}$I labeled factor X revealed factor Xa generation. Factor X activator was characterized as a serine protease and the molecular weight was found to be 66 kD. Immunohistochemical studies using H4 localized factor X activator to the glomerular mesangium and capillary wall of 4 to 6 month old diseased MRL/lpr mice. There was a correlation between the expression of glomerular factor X activator, severity of nephritis and the presence of fibrin in the glomeruli. Furthermore, immunogold labeled H4 bound to the dense deposits in the sub-endothelial and mesangial regions as well as to the macrophages and altered endothelial-like cells of diseased glomeruli.
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### ABBREVIATIONS

<table>
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<tr>
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<tr>
<td>anti-dsDNA</td>
<td>Anti-double stranded DNA</td>
</tr>
<tr>
<td>anti-ssDNA</td>
<td>Anti-single stranded DNA</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>E-64</td>
<td>Trans-epoxysuccinyl-L-leucylamido (4-guanidino)-butane</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GBM</td>
<td>Glomerular basement membrane</td>
</tr>
<tr>
<td>GN</td>
<td>Glomerulonephritis</td>
</tr>
<tr>
<td>gld</td>
<td>Generalized lymphoproliferative disease</td>
</tr>
<tr>
<td>gp70</td>
<td>Glycoprotein 70</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>H4</td>
<td>Monoclonal antibody against factor X activator</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>LA</td>
<td>Lupus anticoagulants</td>
</tr>
<tr>
<td>lpr</td>
<td>Lymphoproliferation</td>
</tr>
<tr>
<td>Lrdm</td>
<td>lpr renal disease modifier</td>
</tr>
<tr>
<td>MHV-3</td>
<td>Murine hepatitis virus 3</td>
</tr>
<tr>
<td>Musfiblp</td>
<td>Mouse fibrinogen-like protein</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Type 1 plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>Procoagulant activity</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethyl sulfonyl fluoride</td>
</tr>
<tr>
<td>RVV</td>
<td>Russell's viper venom</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td><strong>Tgfb</strong></td>
<td>T cell growth factor B</td>
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<tr>
<td>---</td>
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</tr>
<tr>
<td><strong>xid</strong></td>
<td>X chromosome-linked immunodeficiency</td>
</tr>
<tr>
<td><strong>Yaa</strong></td>
<td>Y-linked autoimmune accelerator</td>
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CHAPTER 1: INTRODUCTION

1.1 Systemic lupus erythematosus (SLE)

SLE is an autoimmune disease with diverse and variable clinical manifestations. The disease may affect any organ in the body, but it is principally characterized by injury to the skin, joints, kidney, lung, heart and blood vessels (1). About 70% of the patients with SLE develop immune complex mediated GN which is associated with high morbidity and mortality (2). Like most autoimmune diseases, SLE is predominantly a disease of women, afflicting females of childbearing age, at a ratio of nine women to each man (3). Autoantibody production and elevated serum levels of anti-DNA antibodies are the hallmark of this condition. SLE, a multifactorial immune disease, is influenced by genetic background, environmental assaults and hormonal modifications (4,5).

The most common immunologic abnormality found in all animal models of lupus as well as human patients is a pronounced B cell hyperactivity (6-9). Although T cells do not play a direct role in SLE, CD4+ T cells seem to be required for the production of pathogenic IgG autoantibodies (10). Hence the development of SLE appears to involve the development of both autoreactive T cells and B cells. There is considerable evidence that the development of SLE has a strong genetic basis and that the breakdown of immunological tolerance is influenced by certain susceptibility genes in either or both lymphocyte populations (11). A number of studies have identified that SLE has a strong genetic basis with contributions from both MHC and multiple non-MHC genes (11-13). Deficiency of classical pathway complement genes has been shown to predispose to SLE (14).
1.2. Murine SLE

The etiopathogenesis of autoimmune diseases cannot be readily studied without the appropriate animal models. Although, SLE-like disease is found in a number of species like the dog (15), the most extensively studied is the mouse, in which a number of lupus-prone strains exist. Murine SLE is a good model of human disease because most of the immunologic abnormalities that are fundamental to the human disease are also operative in the mouse (16).

Murine models of SLE include the classical NZB/WF₁ (female), BXSB (male), and the MRL/lpr (17). In these strains, the genetic background predisposes the animals to a disease that becomes clinically manifest and then fatal in the second year of life. However, in each of these strains, there are unique accelerating factors that when acting on the lupus-prone genetic background cause a conversion of the late-life disease to an early-life disease that becomes clinically manifest in the first few months of life and then fatal within 5-7 months (17). The accelerating factors are female hormones in the NZB/WF₁ strain, the autosomal recessive lymphoproliferation (lpr) gene in the MRL and the Y linked autoimmune accelerator gene (Yaa) in BXSB. Other less studied murine strains that develop SLE-like disease are the Palmerstrom-North (18) and the Motheaten (19) mice.

Murine SLE is caused by genetically determined abnormalities of the hematopoietic stem or lymphoid precursor cells and not by abnormal autoantigens (20). Studies have shown that allogeneic bone marrow transplantation can prevent the development and progression of disease in MRL/lpr and BXSB mice (21). There is a generalized B cell hyperactivity leading to hypergammaglobulinemia, early IgM to IgG switching, production of autoantibodies against a variety of endogenous antigens, formation of immune complexes and a variety of histopathologic manifestations, including glomerulonephritis, myocardial infarcts and arthritis (16).
A number of in vivo studies with anti-T cell antibodies (22) and in vitro studies on signal requirements for lupus B cell activation/differentiation (23,24) have indicated that T cells are required for autoantibody production. However, only in the MRL/lpr mice the physical presence of the thymus is critical, although the genotype of the thymus is irrelevant (25). The major mechanism responsible for B cell hyperactivity may be B cell hyperresponsiveness to and/or hyperproduction of, B cell proliferation and differentiating cytokines derived from accessory cells (23,24).

1.3. The MRL/lpr murine model of SLE

The MRL (H-2k) strain was developed in 1976 by Murphy and Roths at the Jackson Laboratories (26). It originated as a series of crosses involving the inbred strains AKR/J, C57BL/6J, C3H/Di and LG/J. The series of crosses were started in 1960 to transfer a mutation for achondroplasia from the high leukemic background of strain AKR to a background without an early incidence of leukemia. In the twelfth generation of inbreeding, some of the offspring developed massive lymph node enlargement while others did not. The two groups of MRL mice were separated, and with subsequent inbreeding, became two sublines of the MRL mice: one called MRL/lpr that expressed lymphadenopathy, and the other called MRL/n that did not express lymphadenopathy. The genomic composition of the MRL mouse is derived 75.0% from LG/J, 12.6% from AKR, 12.1% from C3H, and 0.3% from C57BL/6 mouse. The MRL/n mouse which lacks the lpr gene has delayed disease with 50% mortality at 476 days for females and 546 days for males. MRL/lpr mice demonstrate 50% mortality at 143 days for females and 154 days for males.

1.3.1. Morphologic manifestations in MRL/lpr mouse

The histopathologic manifestations of murine SLE (MRL/lpr mouse in particular)
include glomerulonephritis, thymic atrophy, myocardial infarction, lymphoid hyperplasia and arthritis.

The most consistent abnormality and the major cause of death in all lupus mice is immune complex GN (17). The MRL/lpr strain develops a subacute proliferative form of GN which leads to renal failure and ultimately causes death. In these mice, the glomerular lesions involve the accumulation of macrophages and proliferation of both mesangial and endothelial cells with occasional crescent formation and basement membrane thickening. Immunofluorescence shows granular deposits of mouse IgG and C3 of variable intensity in both the capillary wall and the mesangium, often predominating in the former where the finely granular deposits are closely approximated (27). The nature of renal immunoglobulin deposits in the MRL/lpr mouse is predominantly of the IgG2a and IgG2b subclass (17).

Another consistent feature of murine SLE is severe thymic cortical atrophy (27). In MRL/lpr mice, thymic atrophy and cystic necrosis appear by 2 months of age and progress to a complete loss of cortical areas by 3.5 months of age. About 15 to 30% of each strain of lupus mice have old and/or acute myocardial infarction involving either ventricle and judged extensive enough to be a contributing cause of death (27). In approximately 75% of older MRL/lpr mice, acute polyarteritis involving mostly medium-sized arteries of the kidney, genital organs and heart occurs.

Marked splenic and lymph node hyperplasia occurs in all murine lupus models (27). All MRL/lpr mice develop massive lymph node enlargement, commencing by 2 months of age and progressing to over 100 times control weights by 4 to 5 months of age (27). The proliferation is mainly lymphocytic, with a mixture of histiocytes, plasma cells and immunoblasts. Immunoperoxidase analysis of lymph node and spleen sections showed that all murine isotypes and IgG subclasses are represented in the plasma cells, but IgG1 and IgG2a containing cells predominate. In one-third to one-half of older, terminally diseased MRL/lpr mice, the larger nodes show extensive hemorrhagic and
cystic necrosis which may account for the clinically evident terminal reduction in lymph node size (27).

Among the lupus mice, 20-25% of old, diseased MRL/\(lpr\) mice have swelling of the joints and surrounding tissues of the hind feet and lower legs (17). The cellular composition of the inflamed synovium of the MRL/\(lpr\) mice includes plasma cells, macrophages, and lymphocytes. The MRL/\(lpr\) mouse represents the only animal with noninfectious, spontaneous arthritis accompanied by rheumatoid factor, and closely resembles humans with rheumatoid arthritis in manifestations including vascular lesions, synovial inflammation, perisynovial and periarticular tissue mononuclear infiltrates, pannus formation, cartilagenous joint surface destruction and joint fusion as a terminal event.

1.3.2. Serologic manifestations in MRL/\(lpr\)

All lupus mice have significantly higher total polyclonal IgG and IgM concentrations than normal mice (28). In the older MRL/\(lpr\) mouse, the IgG subclasses that predominate are IgG\(_1\), IgG\(_2\)a and IgG\(_2\)b. The switch of immunoglobulin from predominantly IgM to IgG heralds the onset of severe disease.

A characteristic feature of human and murine SLE is the presence of antibodies to a variety of nuclear antigens (29). Studies show that anti-nuclear antibodies are mainly directed against DNA, RNA-protein complexes, or histones. In murine SLE, none of the affected strains show significant levels of anti-double stranded DNA (anti-ds DNA) antibodies at 2 months of age, however at 4-5 months of age all SLE mice show elevated levels of these antibodies. Antibodies to single-stranded DNA (anti-ss DNA) are found at low levels in normal mice. The concentration of this antibody increases in 2 months old lupus mice and it reaches even higher levels at 4 months of age. In MRL/\(lpr\) mouse, in addition to anti-DNA autoantibodies, anti-Sm autoantibodies and antibodies to Z-DNA
("left handed" DNA) are observed (30,31). Lupus mice also contain antibodies to gp70 (glycoprotein found in serum and has structural and immunologic similarity to retroviral envelope protein) (27). Even though similar levels of gp70 are found in the serum of both normal and lupus mice, only lupus mice develop antibodies against this protein and contain gp70-anti-gp70 complexes in the serum(32). The amount of gp70-anti-gp70 complexes increase with the progression of disease in all lupus mice.

In all three strains of lupus mice, the concentration of hemolytic complement falls with the onset or progression of disease (27). Increased levels of cryoglobulins correlate with age and disease severity (27). In addition, the MRL/lpr mice develop IgM and IgG rheumatoid factors at approximately 3 to 4 months of age. (27).

1.3.3. Genetic analysis of MRL/lpr mice

The lpr mutation, a mouse autosomal recessive mutation, was first described in the MRL/lpr mouse (26,27). The lpr mutation arises as the result of insertion of an endogenous retrovirus in the second intron of the Fas gene in MRL/lpr mouse (33-35). This mutation leads to abnormal transcription and splicing of Fas and results in decreased amounts of normal Fas transcripts (34). The lpr mutation produces a massive enlargement of lymph nodes with the expansion of an unusual subset of T cells that are phenotypically Thy-1 CD3+ CD4- CD8+, but express the B220 antigen characteristic of B cells (36-38). Even though the double negative T cells in MRL/lpr mice do not appear to have a functional role, the accumulation of these cells suggests that other T cells escape the normal selection process as well. During normal T cell development, both positive and negative selection takes place to eliminate potentially self-reactive cells (39). Normally Fas gene is expressed in lymphoid and non-lymphoid cells, and the Fas protein is involved in regulating apoptosis. Fas protein is expressed in high levels in CD4+ CD8+ immature thymocytes and in activated mature T and B lymphocytes in normal mice, but not in MRL/lpr mice (40). It is possible that the abnormal regulation of apoptosis due to the lack of functional Fas in the MRL/lpr mouse could result in a failure of thymic
selection, survival of self-reactive T cells and an extension of the life span and prevention of antigen-induced apoptotic death of self-reactive B cells.

The involvement of apoptosis related genes in the development of murine lupus was further supported by the generalized lymphoproliferative disease (gld) mutation. The gld mutation affects the ligand for the fas receptor due to a point mutation in the putative binding site (41). Homozygous lpr mice are phenotypically identical to mice homozygous for the gld mutation (42). The lpr or gld mutation in B6/lpr and C3H/lpr mice, lacking an apparent SLE background, induces the formation of various autoantibodies, but the histological manifestations of SLE are much less evident (43,44). These findings clearly illustrate that in addition to the lpr or gld mutation, other abnormalities are needed for the full manifestation of SLE.

MRL/lpr mice produce large amounts of autoantibodies, and develop massive lymphadenopathy, severe immune-complex nephritis and synovitis. However, MRL/n mice without the lpr mutation develop a late disease, suggesting that lpr accelerates rather than causes the disease (16). This notion is further supported by the observation that lpr congenic mice (mice carrying lpr mutation on different genetic background) produce autoantibodies, but the histological manifestations of SLE are much less evident (16,43). These findings indicate that the manifestations of generalized autoimmune disease in this model result from complex genetic interactions. The ability to define markers at 10 to 20 cM intervals throughout the genome had identified two loci as probable disease modifiers. Genes determining the severity of nephritis in mice expressing the lpr mutation have been mapped to chromosomes 7 and 12, tentatively named Lrdm-1 and Lrdm-2 (lpr renal disease modifier), respectively (45). This analysis indicated that over 50% of the variance in renal disease is accounted for by these two chromosomal regions. Two potential renal disease modifiers have been mapped close to Lrdm-1 (46) and these are Otf-2, an immunoglobulin transcriptional regulator (47), and T cell growth factor B1 (Tgfβ1), a mediator of inflammation and peripheral tolerance (48). Another potential renal disease modifier, Tgfβ3, has been mapped close to the region of Lrdm-2 (49).
Another important factor in the pathogenesis of lupus nephritis could be controlling the quality of autoantibodies with immunopathological consequences. Studies have shown that spontaneous production of rheumatoid factors with cryoglobulin activity was frequently found in the MRL/lpr mouse, but rarely in non-autoimmune mice bearing the lpr or gld mutation (50). Murine IgG3 antibodies exhibit a cryoglobulin activity because of their unique physiochemical characteristics of the gamma 3 constant region (51). A significant fraction of IgG3 monoclonal autoantibodies derived from MRL/lpr mice are highly nephritogenic, producing "wire-loop"-like glomerular lesions (52,53). Studies have shown that interferon-gamma (IFN-γ) can upregulate the production of IgG3, while interleukin 4 (IL-4) can down-regulate the production of IgG3 (54,55). IFN-γ plays an essential role in the progression of murine SLE (56) and its accelerating effects could be due to its immune potentiating activity and enhanced production of IgG3 autoantibodies that are highly pathogenic. Development of lupus nephritis in lupus-prone mice is greatly retarded by the presence of xid (X chromosome-linked immunodeficiency) gene, which causes a selective defect of IgM and IgG3 synthesis (57). Thus, abnormalities in the genes regulating the synthesis of IgG3 autoantibodies could be an important genetic factor in the pathogenesis of murine lupus.

1.4. Lupus nephritis: an immune complex mediated disease

The occurrence of diseases due to immune complex deposits was suspected as early as 1911 by a physician named Clemens Von Pirquet who correctly diagnosed serum sickness as immune complex mediated disease (58). Lupus nephritis is the prototype of diseases caused by the deposition of immune complexes.

The presence of anti-DNA antibodies is a key feature of SLE and their presence correlates with nephritis in both humans and mice (27,59). Both anti-ds DNA and anti-ss DNA antibodies have been associated with lupus nephritis, however the clinical correlation with renal disease appears to be stronger for anti-ds DNA antibodies (60-62).
The association between anti-DNA antibodies and lupus nephritis is supported by the findings that anti-DNA antibodies are concentrated in murine and human glomeruli in vivo and administration of DNA to autoimmune mice speeds up the progression of nephritis (61-63). Furthermore, administering anti-DNA monoclonal antibodies or bacterial DNA alone induces nephritis in non-autoimmune mice (64-66).

Antibodies other than anti-DNA antibodies may also contribute to the onset or progression of lupus nephritis. Anti-chromatin antibodies in general (including but not exclusively anti-DNA antibodies) may contribute to the pathogenesis of lupus nephritis (61,67,68). Recently, characterization of glomerular binding by autoantibodies in the serum of the MRL/lpr mouse has revealed that these antibodies consist of a family of antibodies which bind to a variety of epitopes on chromatin (69). Autoantibodies to gp 70 and to a variety of glomerular basement membrane (GBM) components including laminin, fibronectin, and collagen have also been associated with lupus nephritis (32,70-72).

Nephritogenic autoantibodies in humans and mice are predominantly of the IgG isotype (61,27). The pathogenicity of these antibodies depends on several features including their cationic charge, isotype, cryoprecipitability and possibly their complement fixing ability (73). One significant feature of nephritogenic murine monoclonal anti-DNA antibodies is the increased number of charged amino acids in the heavy chain complementarity-determining regions (CDRs), particularly CDR3, which enhances the binding of antibody to DNA (64).

1.4.1. Mechanisms of immune deposit formation

Genetic factors play a critical role in determining the incidence and severity of glomerular disease (74). Three mechanisms have been proposed to explain the way immune deposits form in glomeruli to cause GN: the circulating immune complex hypothesis, the cross-reactive antibody hypothesis, and the planted antigen hypothesis
The three hypothetical mechanisms by which autoantibodies can cause nephritis are not mutually exclusive and potentially all may contribute to the pathogenesis of nephritis.

The circulating immune complex hypothesis is the oldest hypothesis and it holds that antibodies and antigens form circulating immune complexes which subsequently bind to the glomerulus. The similarity of SLE to experimental immune complex diseases and the detection of circulating immune complexes in both murine and human lupus provide evidence to support this hypothesis (76). The existence and role of DNA/anti-DNA complexes is controversial because even if such complexes exist, they are cleared by the liver rapidly and they bind poorly to the GBM (77-79).

The basis for the cross-reactive hypothesis is that autoantibodies, particularly anti-DNA antibodies, are broadly cross-reactive and can bind to a range of antigens such as GBM components. Most of the experimental evidence for this hypothesis comes from monoclonal antibody studies which suggested that anti-DNA antibodies could bind to glomeruli or components of GBM directly (80,81). The cross-reactive hypothesis suffers from a number of theoretical and technical concerns. Studies clearly illustrate that autoantibody production has the properties of an antigen-driven response and that the antibodies produced are of high specificity and avidity (82,83). Furthermore, many monoclonal anti-DNA antibodies may be anti-nucleosomal antibodies that bind avidly to DNA in vitro by virtue of histones to which they are pre-complexed (84). Finally, the binding of autoantibodies to cultured cell lines or antigens isolated from biologic sources may be mediated by nuclear antigens bound to the cell or protein of interest (85,86). Therefore, the binding of autoantibodies to non-nuclear antigen epitopes on the GBM or glomerulus appears to be a less likely event.

The planted antigen hypothesis holds that autoantigens deposit in the glomerulus and subsequently bind autoantibodies forming immune complexes in situ. The avidity of histones and nucleosomes for GBM proteins provides direct support for this hypothesis. Histones bind well to GBM in vitro and in vivo and this binding may be mediated by
heparan-sulfated glycosaminoglycan, type IV collagen, or anionic phospholipids (84,87,88).

1.4.2. Sites of glomerular immune deposit formation

Immune complexes and complement proteins have long been known as essential factors in producing local tissue damage in lupus. Immune deposits may form in the subepithelial space, sub-endothelial space, the GBM itself, or the mesangium. The location of immune complex deposition and the composition of immune deposits determine the functional and histologic lesions that result and also the type of inflammatory mediators that are activated (89). Sub-endothelial and mesangial deposits are associated with proliferative glomerular lesions, while sub-epithelial deposits are characteristic of membranous GN. Glomerular pathology in SLE is often complex and a broad range of glomerular lesions is observed.

Deposits in the sub-epithelial space are formed primarily by in situ mechanisms and the antigen responsible can be an endogenous, insoluble fixed renal antigen or an exogenous, soluble antigen (eg. DNA) which is deposited in the glomerulus on the basis of some physiochemical characteristics (90). Sub-epithelial deposition of circulating immune complexes is not possible because large complexes cannot pass through the GBM. Sub-epithelial immune complexes stimulate the production of basement membrane components at sites where the epithelium remains attached to its basement membrane. Sub-epithelial deposits are associated with injury to the epithelial cell which is characterized by retraction and fusion of foot processes leading to distortion of the slit diaphragms. This process is complement dependent, being mediated by the generation of the membrane attack complex (90).

Sub-endothelial deposits are usually seen in association with mesangial deposits, and deposits at these sites probably arise by similar mechanisms. These deposits may form by in situ mechanisms and the targets for circulating antibodies are endogenous or
exogenous antigens that are planted at these sites by means of their size, charge, and/or affinity characteristics (91). For example, larger or anionic antigens are more likely to be involved because they are less likely to cross the GBM and enter the sub-epithelial space. Deposits at these sites can also result from the entrapment of circulating immune complexes (92). Complexes at these sites have access to the systemic circulation, since they are proximal to the GBM. Immune complexes and complement activation can attract neutrophils, platelets and macrophages, leading to marked inflammation. Other mediators of injury include the activation of the coagulation system and the damaged glomerular endothelium which can be an active participant and regulator of the inflammation (90).

1.4.3. Mediation of immune complex - induced glomerular injury

The nature of the lesion produced depends on the site and composition of the immune deposits. Injury to the glomerulus by any mechanism may result in increased release of factors with inflammatory, mitogenic and procoagulant activities. Structural manifestations of glomerular injury include GBM thickening, cellular proliferation, necrosis, thrombosis, crescent formation, and sclerosis. Functional manifestations of glomerular injury include changes in glomerular filtration rate and glomerular permeability (proteinuria) (90,93).

A number of possible mechanisms of glomerular injury in immune complex GN has been proposed and they are depicted in figure 1.1. Glomerular damage sufficient to cause proteinuria can result by deposition of antibodies alone without the involvement of complement and inflammatory cells. This is usually mediated by the effect of the antibody or immune complexes on epithelial cells (94). Another mechanism is through the activation of complement. The formation of membrane attack complex (C5b-9) may cause damage in a number of ways. It may stimulate epithelial cells to produce increased amounts of normal extracellular matrix components which results in abnormal structure
of the GBM with alterations in permeability (95). It may also cause injury through the activation of epithelial cells with the release of toxic inflammatory mediators (96). The other mechanism by which activation of complement may produce injury is through the recruitment of inflammatory cells (97). Accumulation of neutrophils occurs following the generation of chemotactic fragment C5a and as a consequence of immune adherence resulting from C3b and Fc receptors and neutrophils (97). Following adherence to immune complexes, neutrophils are activated and that leads to the release of proteases and oxidants which produce tissue injury (98). Platelets may also be involved in mediating injury by producing injurious substances (99).

The accumulation of macrophages into the glomerulus may be mediated by their Fc receptors (100). They may mediate tissue injury by producing oxidants, procoagulants, complement factors, cytokines and proteases capable of degrading extracellular matrix (101). Proliferation of mesangial cells is a common histologic feature of many forms of GN. Mesangial cells in culture are known to produce a variety of inflammatory substances and some of these can be produced in response to activation by immune complexes and complement (102). A role for T cells in glomerular injury has been suggested because T cells accumulate in glomeruli in antibody-mediated human and experimental GN (103,104). The mechanism by which they contribute to disease is not clear, it may be through their effects on macrophages and/or mesangial cells.
Immune response to fixed or planted glomerular antigens

Glomerular IC formation

Complement

C5b-9 MAC

C5a/C3b

Epithelial cells

Platelets

Neutrophils

Macrophages

Mesangial cells

Sensitized cells

Proteases &/or Oxidants

Increased glomerular permeability and Proteinuria

Figure 1.1: Schematic depiction of the pathways by which immunologic mechanisms lead to glomerular injury in GN. Activated inflammatory or structural glomerular cells by one of the above mechanisms cause glomerular damage via the release of reactive oxygen species and proteolytic enzymes. (Figure adapted and modified from 105).
1.5. Coagulation and SLE

Activation of the coagulation system in SLE has been implicated by Hardin et. al. who demonstrated increased levels of fibrinopeptide A in patients with active SLE (106). Patients with SLE have a higher incidence of arterial and venous thrombosis (107).

1.5.1. Lupus anticoagulant and SLE

About 10% of patients with SLE have at least one thrombotic event. However, the prevalence of thrombosis increases to 50% in patients with anti-phospholipid antibodies or lupus anticoagulants (108). Lupus anticoagulants (LA) are antibodies with immunologic specificity to anionic phospholipids involved as active sites in the calcium-mediated binding of vitamin K dependent coagulation factors (109,110). Evidence indicates that LA may induce thrombosis by the inhibition of protein C activation system (111). These antibodies prolong clotting time in vitro by inhibiting prothrombin activation, whereas in vivo, the predominant effect might be inhibition of protein C activation, which would favor thrombosis. Also, effects of the antibody on endothelial cells and platelets, or on the fibrinolytic system may all account for thrombosis in vivo (111).

1.5.2. Fibrin deposition in glomerulonephritis

Formation of fibrin is an important event in inflammatory reactions. The indication that coagulation might be an important mediator of glomerular injury came first from the observations that in some cases of crescentic GN in man and experimental animals, material with the staining properties of fibrin could be found in the glomeruli (112). Fibrin deposition is a feature of animal and human GN and has been observed in glomeruli with both endocapillary and extracapillary proliferative lesions (113-115). It is
prominent in those forms of GN with the worst prognosis and the greatest evidence of glomerular damage (113,115). Glomerular fibrin deposition may be found in the capillaries, mesangium, and outside the glomerular tufts in Bowman's space (116). Deposition of fibrin within the Bowman's space is a feature of proliferative GN with crescent formation. Endocapillary proliferation in response to fibrin deposition in Bowman's space has been used to explain crescent formation.

There are a number of possible mechanisms by which fibrin may cause tissue injury. Fibrin could cause tissue damage by interfering with the microcirculation. Fibrin deposits may impair macrophage mobility and seclude soluble mediators of inflammation (117,118). Fibrin degradation products are cytotoxic to mesangial cells and they can attract circulating inflammatory cells. The relevance of fibrin deposition to glomerular injury has been emphasized by defibrination which reduced glomerular damage in both animals and humans (119-121).

The mechanisms leading to fibrin deposition in GN are unclear. Deposition of fibrin occurs as a result of an imbalance between the formation and degradation of fibrin. Possible mechanisms leading to formation of fibrin include the activation of Hageman factor by immune complexes, activation of coagulation by damaged GBM, endothelial cells and complement and the release of procoagulants from macrophages, intrinsic glomerular cells and platelets which could lead to the activation of coagulation (122,123). Another possible mechanism is the failure of removal of fibrin deposits either due to inhibition of fibrinolysis or due to reticuloendothelial dysfunction (123). Figure 1.2 outlines the major factors and cofactors involved in the coagulation and fibrinolytic pathways.
Figure 1.2: Coagulation and fibrinolytic system. The three cellular procoagulants are circled. The abbreviations used in this figure are: PK (Prekallikrein), HK (High molecular weight Kininogen), PL (Phospholipids) and t-PA (tissue plasminogen activator). Figure adapted and modified from 124.
The traditional view is that immune injury activates the intrinsic pathway by exposure of damaged tissue membrane. Hageman factor, factor XII, after contact and binding to sites of endothelial damage, complexes with high molecular weight kininogen, factor XI, and prekallikrein, resulting in the proteolytic activation of the latter three molecules.

There are many observations that are not consistent with the traditional view. There was no participation of factor VIII in fibrin-associated GN (114), and in experimental models of GN, glomerular accumulation of fibrin occurred before factor VIII can be detected in glomeruli. Finally, in rats congenitally deficient in prekallikrein and high molecular weight kininogen, glomerular fibrin deposition is similar to that seen in normal rats (125). Taken together, these findings indicated that intrinsic pathway activation alone could not account for glomerular fibrin deposition in the various forms of GN. Previous studies have also indicated that platelets do not have a significant role in deposition of fibrin in experimental anti-GBM GN (126).

Another potential trigger for fibrin deposition is the increased expression of glomerular procoagulant activity which has been found in nephrotoxic nephritis, acute serum sickness, mercuric chloride-induced GN and human proliferative GN (127-130).

1.5.3. Cellular origin of glomerular PCA

The cellular origin of glomerular PCA may be from blood borne or intrinsic glomerular cells. The major infiltrating cells are neutrophils, lymphocytes, and monocytes/macrophages. Among these, neutrophils and lymphocytes do not produce PCA (131), whereas monocytes/macrophages are a potent source of various forms of cellular PCA. The association of macrophages, enhanced PCA, and glomerular fibrin deposition has been reported in human and experimental GN (127-130). Structural glomerular cells may respond to inflammatory mediators, such as antibodies,
complement, or immune complexes, cytokines or may be stimulated by inflammatory cells to express PCA. Cultured mesangial cells have been shown to produce PCA which is upregulated in response to tumor necrosis factor (TNF) and lipopolysaccharide (132). Endothelial cells have also been shown to produce increased amounts of PCA in response to cytokines, such as TNF and interleukin 1 and immune complexes (133).

1.5.4. Nature of PCA

Three distinct cellular forms of PCA have been described so far: tissue factor (134,135), direct factor X activator (136), and a direct prothrombinase (137,138). Other procoagulants consist of complexes of components of the coagulation cascade (139,140). The nature of these procoagulants can be identified by their size, and their dependence on specific coagulation factors for their activity.

Tissue factor has been identified as the primary initiator of coagulation in vivo. It is a transmembrane glycoprotein that serves as a high-affinity cell surface receptor and cofactor for factor VII (134). The tissue factor-factor VIIa complex activates factors IX and X. Tissue factor can be produced by a wide variety of cells including macrophages and endothelial cells (141). It is usually sequestered from contact with other coagulation factors in the plasma. Macrophages and endothelial cells can be induced to express tissue factor in response to endotoxin, cytokines, immune complexes, T lymphocytes and complement components. Tissue factor is a 31 kD protein that when glycosylated exhibits a size of approximately 46 kD (142). In a number of experimental models of GN including nephrotoxic nephritis, acute serum sickness and anti-glomerular basement membrane antibody induced GN in rabbits, expression of glomerular PCA was increased. The increase in glomerular PCA in these models was coincident with the accumulation of macrophages in the glomeruli (127-130). Glomerular PCA in these models had characteristics of tissue factor in that for the expression of glomerular PCA, factors VII and V were required, but not factors VIII and XII (127). Furthermore, the glomerular
PCA was inhibited by phospholipase C and concanavalin A, indicating that phospholipids and glycoside residues are essential for its functional activity.

A number of factor X activators have been described to date. Factor X activators require the presence of coagulation factors X, V, and II for their activity. Gordon and co-workers have identified a factor X activator, a cysteine protease, in extracts of human and animal tumors and in cultured malignant cells (143). Production of another factor X activator by methylcholanthrene - induced rat fibrosarcoma has also been described (144).

Murine monocytes/macrophages express a direct prothrombinase, mouse fibrinogen-like protein (musfiblp), in response to murine hepatitis virus 3 (MHV-3) infection (145). The expression of this PCA does not require the presence of coagulation factors XII, VII, X or V, but does require prothrombin to convert fibrinogen to fibrin (138). The size of this molecule was found to be approximately 70 kD. Several lines of evidence imply that activation of the coagulation system by MHV-3 plays a critical role in the pathogenesis of MHV-3 induced fulminant hepatitis. First, induction of musfiblp correlates well with the severity of disease (146). Second, administering exogenous prostaglandin E2 inhibits the induction of PCA and prevents the development of hepatic necrosis (147). Finally, it was shown that when mice were treated with a monoclonal antibody to the MHV-3 induced PCA, survival increased (148).

1.5.5. Lupus nephritis and PCA

There is evidence that coagulation plays a role in mediating glomerular damage in lupus nephritis (106,149-151). Kant et al., had reported that glomerular thrombi occur frequently in lupus nephritis and that glomerular thrombosis and sub-endothelial deposits were better predictors of glomerular sclerosis than hypercellularity, necrosis and crescent (152). In a study by Cole et al., PCA was measured in circulating mononuclear cells from patients with SLE. It was found that monocyte PCA directly correlated with
endocapillary proliferation in patients with lupus nephritis (153). In another study, they also showed that in male BXSB autoimmune mice, plasma stimulated lymphocytes induced splenic macrophage PCA, which occurred in concert with the evolution of glomerular disease (154). Finally, the relevance of fibrin deposition to glomerular injury in murine lupus nephritis was shown by defibrination with ancrod which delayed the development of renal fibrin deposition and GN and improved survival (121).

1.6. Hypothesis

My hypothesis is that increased expression of a glomerular factor X activator in MRL/lpr mouse leads to the deposition of fibrin in the glomeruli and hence plays an important role in the progression of lupus nephritis.

1.6.1. Objectives

The objectives of this thesis can be broadly identified as follows:

- determine if there is indeed a relationship between the expression of glomerular PCA and onset of disease in lupus-prone mice. If there is a relationship then assess the correlation between the extent of glomerular injury, fibrin deposition and the expression of glomerular PCA.

- identify the nature of the glomerular PCA and use a monoclonal antibody produced against the glomerular PCA to characterize it.

- localize the expression of glomerular factor X activator to identify the cellular source.
1.7. Scope of the Thesis

This thesis describes the characterization and localization of a glomerular procoagulant activity in lupus nephritis using the MRL/lpr mouse.

In chapter 2, glomerular PCA in normal mice (Balb/cJ) was compared to that in lupus-prone mice. In older lupus mice, there was a significant augmentation in glomerular PCA when compared to the normal mice. This activity was characterized as a factor X activator in MRL/lpr mice using plasma that was deficient in various coagulation factors. This was further confirmed by performing radiolabeled factor X cleavage assays. The monoclonal antibody developed against glomerular factor X activator (H4) bound to a 66 kd protein in the glomeruli. The factor X activator was further characterized as a serine proteinase.

In chapter 3, the correlation between the presence of factor X activator, fibrin deposition and the extent of glomerular damage is described. Immunofluorescence studies revealed that factor X activator was present in the mesangium and capillary walls of the glomeruli of diseased MRL/lpr mice. There was no staining in control animals or in any of the other organs of the affected MRL/lpr mice. The majority of the mice with severe glomerular lesions had both factor X activator expression and fibrin deposition in the glomeruli. Immunogold staining revealed the presence of factor X activator in the mesangial and sub-endothelial dense deposits and in macrophages and altered or activated endothelial-like cells.

These findings allow us to conclude that in MRL/lpr mice with lupus nephritis, factor X activator expression is upregulated in the glomeruli and this might lead to the deposition of fibrin in the glomeruli which in turn could play a critical role in the development of injury in this model of lupus nephritis (Chapter 4).
CHAPTER 2: IDENTIFICATION OF A UNIQUE GLOMERULAR FACTOR X ACTIVATOR IN MURINE LUPUS NEPHRITIS

2.1. Purpose

In order to gain understanding of the relevance of local activation of coagulation in the renal disease in lupus, the current studies were designed to assess and characterize glomerular PCA before and after the development of murine lupus nephritis.

2.2. Materials and Methods

2.2.1 Mice and virus

Male BXSB, Female NZBxWF1, female MRL/lpr, and male Balb/cJ mice, 6 to 8 weeks and 4 to 5 months of age and A/J and CAF1 mice, 6 to 8 weeks 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 and at the animal facilities at Toronto Hospital.

Mouse hepatitis virus strain 3 (MHV-3) was plaque purified on monolayers of DBT cells and grown to titers of $1.0 \times 10^7$ PFU/ml in 17 CL1 cells. Viral titers were determined on monolayers of L2 cells in a standard plaque assay (145).

2.2.2 Cells

Peritoneal macrophages were harvested from Balb/cJ mice 4 days after injection with 2 ml of 3% thioglycolate (Difco, Detroit, Michigan, USA) as described previously.
After being washed, cells were resuspended at $2 \times 10^6$ /ml in RPMI 1640 (ICN Biomedicals Inc., Costa Mesa, California) supplemented with 2 mM glutamine (Sigma Chemical Co., St. Louis, MO) and 2% heat-inactivated fetal calf serum (Gibco, Burlington, Ontario, Canada). Viability exceeded 95% by trypan blue exclusion. Macrophages were stimulated either with 10 µg of lipopolysaccharide (LPS) (Sigma, St. Louis, Missouri, USA) or with MHV-3 at a 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).

### 2.2.3 Isolation of glomeruli

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 majority of medulla removed and discarded. The remaining tissue (cortex) was minced and subsequently passed through a stainless steel sieve fitted with a 230 µm sieve (EC Apparatus Corporation, VWR Scientific Canada Ltd., London, Ontario, Canada). The sieve was washed and the tissue 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 10cc 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 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 aliquotted 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).

2.2.4 Measurement of PCA

Samples were assayed for PCA by their ability to accelerate the spontaneous clotting time of normal recalcified platelet-poor human plasma as previously described (131). For determining PCA, 300 glomeruli/assay or 20 000 macrophages/assay were used. To the lysate, 100 μl of 25 mM CaCl₂ that was warmed at 37°C was added, followed by 100 μl of decalcified platelet-poor normal human plasma. The time in seconds for the appearance of fibrin gel was recorded. To establish units, a rabbit brain thromboplastin standard at 36 mg dry mass per milliliter (Sigma) was assigned a value of 100,000 mU. Then log dilutions of this standard were used to derive a standard curve. The medium and reagents were without activity. Additional PCA assays were performed with human plasmas which were deficient in coagulation factors II, V, VII, VIII, IX, X, XI, XII (Helena Laboratories, Beaumont, Tex.) to determine the nature (factor dependence) of the glomerular PCA.

2.2.5 Glomerular Lysate-Mediated Cleavage of Factor X and prothrombin

Factor X and prothrombin (Sigma) were radiolabeled with ¹²⁵I enzymatically using immobilized lactoperoxidase and glucose oxidase (Enzymobeads: Bio Rad Laboratories, Mississauga, Ontario) according to the product information. For assessment of cleavage of factor X, 0.06 ng of factor X plus 20 μl of 25 mM CaCl₂ in 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.8, and either 0.02 ng of Russell’s viper venom (RVV, Sigma Chemical Company, St. Louis, Missouri, USA) or a lysate from 300 glomeruli were mixed. To study cleavage of prothrombin, 72 ng of ¹²⁵I prothrombin was added to 0.6 ng of factor X, 20 μl of 25 mM CaCl₂, and either 0.6 ng of RVV or a lysate
of 300 glomeruli. All samples were incubated at 37°C for up to 60 minutes. The reaction was terminated by adding 10% sodium dodecyl sulphate and 10% EDTA, both to a final concentration of 1%. Thereafter, samples were loaded on to 10% sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoresis carried out according to the method of Laemmli (155). Following electrophoresis, the gels were fixed, dried and analysed by autoradiography for 125I labeled factor X and prothrombin and their cleavage products.

2.2.6 Inhibition of glomerular PCA by protease inhibitors

To further characterize glomerular PCA, glomerular lysate (300 glomeruli/assay) or LPS stimulated macrophages (20 000 cells/assay) were incubated with Concanavalin A (Con A) at 10 μg/ml, aprotinin at 50 μg/ml, phenylmethyl sulfonyl 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 Chemical Co., St. Louis, MO) or buffer for 30 minutes at 37°C, and then assayed for PCA as described above. Glomeruli with buffer instead of inhibitors was used as a control.

2.2.7 Production of monoclonal antibody against glomerular PCA

MRL/lpr mouse glomeruli were isolated from five month old mice (those with severe GN and expressing high glomerular PCA) as described above. About 20 to 30 μg of glomerular protein was mixed with 100 μl of killed Bordetella pertussis (2 x 10⁹ cells) and injected into the peritoneal cavity of A/J mice. Fourteen and twenty one days later, animals were boosted intra-peritonealy with glomerular proteins expressing high PCA. On day 35, the animals were boosted one additional time with glomerular protein, and 24 hours later spleens were removed. Fusion of spleen cells to the SP2/0 myeloma cell line and isolation of hybridomas were performed as described (156). Screening and selection
of the hybridomas were done by inhibition of glomerular PCA using the one stage clotting assay described below. The supernatant from each hybridoma well was collected and used to check their ability to inhibit glomerular PCA as described below. Each hybridoma was subcloned at least three times.

Tumors were then grown by intra-peritoneal injection of $1 \times 10^6$ hybridoma cells into CAF1 mice previously primed with 0.5 ml of Pristane (Sigma). Ascites was harvested after 10 to 14 days and the class of monoclonal antibody was determined to be IgM by Ouchterlony (157). The monoclonal antibody developed against glomerular factor X activator was named H4 and the class and some other characteristics of H4 were further confirmed in a dot blot. For this purpose, H4 was radiolabeled with $^{125}$I and checked to see its ability to bind to goat anti-mouse IgM and IgG and normal mouse IgM and IgG. It was found that H4 only bound to goat anti-mouse IgM. H4 was purified from ascites using the EZ-sep$^R$ ascites purification kit (Pharmacia LKB Biotechnology Inc, Uppsala, Sweden) and stored in a 0.01 M carbonate buffer, pH9.0.

### 2.2.8 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 minutes at room temperature. Then the PCA was measured as described above by adding 100 μl of the incubation mixture to 100 μl of normal citrated human plasma and 100 μl of 25 mM CaCl$_2$. The ability of normal mouse IgM to inhibit glomerular PCA was assessed as described for H4. RPMI-1640 instead of antibody was used as another control. The effect of H4 on LPS stimulated macrophages was also studied.
2.2.9 Western blotting with H4

Proteins were extracted from 3 000 glomeruli, 1.0 x 10^6 MHV-3 stimulated Balb/cJ macrophages, or 1.0 x 10^6 LPS stimulated Balb/cJ macrophages with a buffer containing Tris-HCl (50 mMol/L, pH 7.5), NaCl (150 mMol/L) (TBS), Nonidet P-40 (1% v/v), and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mMol/L PMSF). Samples were subjected to 10% SDS-PAGE and electrophoresis carried out according to the method of Laemmli (155), followed by transfer to nitrocellulose by the method of Burnette (158). The membrane was then blocked with 4% non-fat milk in a buffer containing 0.1% Tween 20 in TBS for 2 hours, reacted with 5 μg/ml H4 overnight at 4 °C and washed 5 times with 0.1% Tween 20 in TBS for 5 minutes each time. The membrane was then incubated for 1 hour with a horseradish-peroxidase-conjugated anti-mouse IgM μ chain specific (Zymed Corp) and washed with 0.1% Tween 20 in TBS five times. The immunoreactive protein bands were visualized using the enhanced chemiluminescence system (Amersham Corp, Arlington Heights, IL). A control blot was performed exactly as described above, but without the addition of H4 to exclude the possibility of non-specific binding due to the second antibody alone.
2.3. RESULTS

2.3.1 Age and glomerular PCA

In order to assess the relationship between age and glomerular PCA in normal and lupus-prone mice, we measured the glomerular PCA of young (6 - 8 weeks) and old (4 - 6 months) mice of male Balb/cJ, female MRL/lpr, female NZB x WF1 and male BXSB. Data illustrating spontaneous PCA of glomerular lysates from young and old mice of several different strains are presented in figure 2.1. In Balb/cJ mice, there was no significant increase in glomerular 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 was performed on the MRL/lpr strain only.

2.3.2 Characterization of glomerular PCA

This study was performed to identify the cellular nature of the activity (tissue factor, prothrombinase or factor X activator) that was responsible for increased glomerular PCA expression. The factor dependence of glomerular PCA and tissue factor was compared using glomerular lysates from 5 month old MRL/lpr mice, LPS stimulated macrophages, and human plasma congenitally deficient in the various clotting factors. Prolongation of the clotting time in plasma deficient in a known factor indicated that the deficient factor was required for the cellular PCA to maximally accelerate coagulation. The data shown in Table 2.1 indicates that factors X, V and II are required for glomerular PCA expression, whereas factors VII, VIII, IX, XI, and XII are not needed. The PCA of LPS stimulated macrophages is due to the expression of tissue factor and this PCA required the presence of factors II, V, VII, and X for its expression. The glomerular PCA does not depend on factor VII for its activity, whereas the tissue factor PCA required factor VII.
2.3.3 Factor X activation

The nature of glomerular PCA was examined further by assessing the ability of glomerular lysates from 5 month old MRL/lpr mouse to cleave radiolabeled factors X and prothrombin directly. The autoradiograph results from factor X activation experiment is presented in figure 2.2. In lane 1, a single high molecular weight species (approximately 70 kD) of intact $^{125}$I labeled factor X was observed after incubation with buffer and calcium. Whereas addition of RVV, a potent activator of factor X, in the presence of calcium produced cleavage products corresponding to the size of known derivatives of activated factor X (lane 2). Incubation of $^{125}$I-factor X with glomerular lysates from MRL/lpr mouse expressing high PCA and calcium generated similar products (lane 3). When incubation of $^{125}$I-factor X with glomerular lysates was carried out in the absence of calcium, there was no observable cleavage of factor X (data not shown).

The ability of glomerular lysate to directly activate prothrombin was also assessed. The results from this experiment is presented in figure 2.3 and in lane 1, a single high molecular weight band (72 kD) of intact prothrombin was observed after incubation with buffer and calcium. Because factor Xa is the physiologic activator of prothrombin, RVV in the presence of factor X and calcium was used to generate factor Xa which in turn can activate prothrombin. It is seen that in lane 2, RVV in the presence of factor X and calcium generated cleavage products of prothrombin. Glomerular lysate was unable to activate prothrombin directly (lane 3). However, the glomerular lysate was able to generate cleavage products of prothrombin when factor X was added to the system, indicating that the glomerular lysate produced factor Xa which in turn activated prothrombin.
2.3.4 Nature of factor X activator

To determine the protease characteristic of the glomerular factor X activator, the ability of different protease inhibitors to inhibit glomerular factor X activator was assessed along with tissue factor and the results are presented in figure 2.4. There was no significant effect of E-64 (cysteine protease inhibitor) on glomerular PCA or TF mediated PCA. Con A did not significantly inhibit glomerular PCA, but inhibited tissue factor PCA by greater than 90%. Aprotinin (serine protease inhibitor) inhibited glomerular PCA by approximately 70% and tissue factor PCA by 50% and PMSF (inhibits serine and some cysteine proteases) inhibited glomerular PCA by 80% and tissue factor PCA by 75%.

2.3.5 Effect of H4 on glomerular factor X activator and tissue factor

The ability of the monoclonal antibody produced against glomerular factor X activator (H4) to inhibit factor X activation was assessed in a one stage-clotting assay. This assay was also performed to assess the specificity of the antibody for the glomerular factor X activator. The H4 inhibition results are presented in Figure 2.5. H4 inhibited glomerular PCA by 70% and did not have a significant inhibitory effect on tissue factor. Normal mouse IgM did not have any effect on factor X activation.

2.3.6 Western blot analysis

Western blot analysis using H4 monoclonal antibody was performed to identify the molecular weight of the factor X activator and these results are presented in Figure 2.6. H4 bound to a protein with molecular weight of 66 kD in glomeruli from a 5 months old MRL/lpr mouse expressing high glomerular PCA. The band was absent in glomeruli from a 2 month old MRL/lpr mouse expressing low PCA. There was no binding to
macrophages expressing tissue factor (LPS stimulated) or direct prothrombinase (MHV-3 stimulated). Furthermore, there was no binding in the control blot with just the second antibody (data not shown).
Figure 2.1: Effect of age on Murine Glomerular PCA. Spontaneous PCA of glomerular lysates (3000 glomeruli) from young and old mice of 4 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 all three lupus strains. Results represent the mean ± SD of 4 experiments done in triplicate. (* indicates P < 0.05)
Figure 2.2: Cleavage of $^{125}$I labeled factor X. An autoradiograph of the cleavage of $^{125}$I labeled factor X when incubated with different constituents. In all cases, (a) represents time 0, (b) 30 minutes, and (c) 60 minutes. Factor X alone (lane 1), factor X + RVV + CaCl$_2$ (lane 2), and factor X + glomerular lysate from MRL/lpr mice + CaCl$_2$ (lane 3). Both RVV and glomeruli cleaved factor X at time 30 and 60 minutes.
Figure 2.3: Cleavage of $^{125}$I labeled prothrombin. An autoradiograph of the cleavage of $^{125}$I labeled prothrombin when incubated with different constituents. In all cases (a) refers to time 0, (b) 30 minutes and (c) 60 minutes. Prothrombin alone (lane 1), prothrombin + RVV + factor X + CaCl$_2$ (lane 2), prothrombin + glomerular lysate + CaCl$_2$ (lane 3), and prothrombin + glomerular lysate + factor X + CaCl$_2$ (lane 4). RVV and glomeruli cleaved prothrombin at time 30 and 60 minutes, only in the presence of factor X.
Figure 2.4: Effect of protease inhibitors on factor X activator and tissue factor. Glomerular lysate (300) or LPS stimulated macrophages (20,000) were mixed with reported amounts of various protease inhibitors or buffer alone for 30 minutes at room temperature. PCA was measured in the one stage-clotting assay. Results are presented as the mean of four separate experiments done in duplicate and are expressed as the percent activity left for each inhibitor relative to buffer, ± SD. (*) represents P < 0.05.)
Figure 2.5: Effect of H4 on glomerular factor X activator and tissue factor.
Glomerular lysate (300) or LPS stimulated macrophages (20,000) were mixed with an equal volume of H4 (4µg) for 30 minutes at room temperature. PCA was measured in a one stage clotting assay. Results represent the mean ± SD of four separate experiments done in duplicate. (* represents P < 0.05).
Figure 2.6: 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 2000 mU/10^5 cells (lane 3), or MHV 3 stimulated macrophages with PCA of 2500 mU/10^5 cells (lane 4) was resolved by electrophoresis, transferred to nitrocellulose and reacted with H4. A band corresponding to molecular weight of 66 kD is observed in the first lane.
Table 2.1
Effect of Factor deficient plasmas on glomerular PCA in MRL/lpr mice and tissue factor

<table>
<thead>
<tr>
<th>Plasma Deficient in factor:</th>
<th>PCA (mU/3000 glomeruli)</th>
<th>PCA (mU/10^6 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>N/D</td>
</tr>
<tr>
<td>X</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>XI</td>
<td>2176 ± 136</td>
<td>N/D</td>
</tr>
<tr>
<td>XII</td>
<td>1750 ± 218</td>
<td>N/D</td>
</tr>
</tbody>
</table>

Glomeruli from MRL/lpr mice and LPS stimulated macrophages were assayed for their ability to shorten the spontaneous clotting time of recalcified normal platelet-poor citrated human plasma or human plasmas deficient in various factors in a one stage clotting assay. Results represent the mean ± standard deviation of three separate experiments.
CHAPTER 3: LOCALIZATION OF GLOMERULAR FACTOR X ACTIVATOR IN MURINE LUPUS NEPHRITIS

3.1 Purpose

This study was undertaken to identify the cellular source of factor X activator and to assess the correlation between the extent of glomerular damage, fibrin deposition and the expression of glomerular factor X activator in murine lupus nephritis.

3.2 Materials and Methods

3.2.1 Histologic assessment of Glomerular Morphology

Old MRL/lpr mice were sacrificed when they had heavy proteinuria. Proteinuria was measured using Albustix® (Bayer Corporation, USA). Kidney cortex of MRL/lpr mice was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm and stained with haematoxylin and eosin (H&E) and picro-Mallory stain.

A light microscopic scoring system was devised to facilitate quantitation of the sequence of glomerular events. Scoring was as follows: - , normal glomerulus; + , predominantly mesangial proliferation; ++ , focal and diffuse endocapillary proliferation with necrosis and/or crescents. Presence or absence of fibrin (from picro-Mallory staining) in glomeruli was noted.

3.2.2 Transmission electron microscopy

Pieces of kidney cortex from 8 old and 2 young MRL/lpr mice were fixed in Universal fixative (159) for 30 to 60 minutes. The tissue was rinsed in 0.1 M phosphate buffer to remove excess fixative, followed by distilled water to remove buffer salts, post-fixed in 2.0% OsO₄ for 30 minutes, and then rinsed in distilled water. Specimens were
dehydrated with graded acetones, infiltrated with Epon/Araldite and polymerized overnight in a 60° C oven. One micron sections were cut and stained with toluidine blue (0.1%) and the region of interest was selected to make the thin sections. The thin sections were cut, placed on grids, stained with Reynold’s lead citrate and alcoholic uranyl acetate and examined in a Philips 201 electron microscope.

### 3.2.3 Immunohistochemistry

In order to localize the expression of glomerular factor X activator, direct immunofluorescence was carried out. For this purpose, H4 (monoclonal antibody against factor X activator produced and characterized as described in chapter 2) and normal mouse IgM were labeled with Fluorescein isothiocyanate (FITC) using CALBIOCHEM® FITC labeling kit (La Jolla, California, USA).

About 2.0 mg of antibody was dialyzed against carbonate buffer to remove free NH\(^{+}\) ions and raises the pH to 9.2. The concentration of antibody was adjusted to 2 mg/ml and 500 µl of 1.0 mg/ml FITC in DMSO was added to the antibody and mixed for 2 hours at room temperature. Unbound FITC was removed from the labeled antibody by passing the mixture through a PD-10 column (Pharmacia), eluting with 0.1 M phosphate buffered saline (PBS, pH 7.2) and collecting 500 µl fractions. The absorbance of each fraction was read at two wavelengths: 280 nm (tyrosine absorption peak) and 495 nm (peak absorbance of FITC conjugated to protein). The fractions that had an absorbance reading between 0.2 to 1.2 at 280 nm were collected and pooled. The degree of labeling achieved (ie. the number of FITC molecules attached to each protein molecule (F:P ratio) was calculated as described below. An F/P ratio of 3 to 5:1 was usually attained for the labeling.

\[
F/P = \frac{(\text{Molecular weight}/389) \times (A_{495}/195)}{[A_{280} - (0.32 \times A_{495})/E^{0.15}]}
\]

(where \(E^{0.15}\) is the \(A_{280}\) reading of a 1 mg/ml solution of the unconjugated protein, as measured in a cuvette of 1.0 cm path length).
Old and young MRL/lpr mice were sacrificed and pieces of kidney cortex, liver, spleen, heart and lung were embedded in OCT compound (Miles) and snap frozen in liquid nitrogen. Four micron sections were cut, fixed in methanol for 5 minutes and blocked with 5% horse serum in PBS for 2 hours. The sections were then stained for two hours with either H4 or normal mouse IgM that was conjugated to FITC. After extensive washing in PBS and 0.05% Tween 20, sections were mounted in glycerol and viewed on a Leitz phase / epifluorescence microscope equipped with x40 Fluotar objective. Photographs were taken on Ektachrome film (Kodak).

3.2.4 Immunogold staining for immuno-electron microscopy

In order to localize the expression of glomerular factor X activator at the ultrastructural level, H4 was labeled with NANOGOLD™ (Nanoprobes, Inc. NY, USA) according to the product information. NANOGOLD™ is a newly developed gold label, prepared using a discrete gold compound rather than colloid. The NANOGOLD™ particle has a reactive sulfo-N-HYDROXY succinimide functionally incorporated into a ligand on the surface of the gold particle; this has specific reactivity towards primary amines, and may be covalently linked to any protein bearing an accessible primary amine. The reagent (30 nmol) as supplied by the company had been lyophilized from 0.02 M HEPES-sodium hydroxide at pH 7.5. It was resuspended in 1.0 ml of deionized water and was added to 1.0 mg of H4 which was dialyzed against 0.02 M HEPES buffer and adjusted to pH 7.5 with dilute sodium hydroxide. The reaction was allowed to proceed at room temperature for one hour and then the unbound gold particles were removed from the antibody conjugate by gel filtration using the PD-10 column (Pharmacia). The conjugate was eluted with 0.02 M sodium phosphate at pH 7.4 with 0.15 M sodium chloride (PBS). The first seven fractions were pooled and the filtration process was repeated again to obtain a higher purity conjugate. The NANOGOLD™ conjugate was stored in PBS with 0.1% bovine serum albumin (BSA) and 0.05% sodium azide at 4 °C.
Since the 1.4 nm NANOGOLD particles are very small, silver enhancement was used to render the NANOGOLD particles more readily visible. The HQ SILVER (Nanoprobes Inc.) used for this purpose, is formulated to give slower, more controllable particle growth and uniform particle size distribution.

3 mm pieces of kidney cortex from 8 old and 4 young MRL/lpr mice were fixed by immersion in 4% paraformaldehyde for 4 hours and then rinsed in 0.1 M phosphate buffer. The tissue was then stored in 0.02M sodium azide in 0.1M phosphate buffer until it was embedded in Lowicryl. Ultra thin sections were cut and mounted on carbon-coated nickel grids. Sections were blocked with 0.15% Glycine in PBS buffer for 3 times and then with 0.5% BSA in PBS for 3 times, 5 minutes each. Grids were then incubated with H4-NANOGOLD™ conjugate diluted 1/20 in PBS-BSA, for 1 hour at room temperature and rinsed in PBS four times, 5 minutes each. They were then postfixed with 1% glutaraldehyde in PBS at room temperature for 3 minutes, rinsed in deionized water four times for 5 minutes each, silver enhanced for 5 minutes with the HQ SILVER® silver enhancement system and rinsed three times in deionized water. Finally, sections were contrasted with uranyl acetate and lead citrate prior to examination under the electron microscope. Controls for this experiment included NANOGOLD labeled anti-mouse IgG and the use of a primary - secondary antibody (actin - NANOGOLD IgG). As a positive control for the technique of gold labeling followed by silver enhancement, we stained all the tissue with anti-murine actin antibody followed by a NANOGOLD conjugated anti-mouse IgG. Sections were also stained with just the second antibody alone.

3.3. Results

3.3.1 Histologic assessment of glomerular morphology

Figure 3.1a shows kidney cortex from a young MRL/lpr mouse that was stained with H&E. There are 4 normal glomeruli in this section. Each glomerulus comprises a tuft of capillaries supported by a mesangial network, enclosed in Bowman's capsule. In the capillary lumens, erythrocytes can be seen. The size, cellularity (number of nuclei),
vascularity (represented by erythrocytes in lumen) and tubules are normal. Figure 3.3a and 3.3b are transmission electron micrographs of a segment of normal glomerulus. At the ultrastructural level, the constituents of the glomerulus are easily seen which include four cellular elements (endothelial cells, mesangial cells, visceral epithelial cells, and parietal epithelial cells) and two extracellular matrix components (GBM and mesangial matrix). The glomerular capillary wall consists of the GBM, lined on its inner side by fenestrated endothelial plasma membranes and covered on its outer aspect by epithelial cell foot processes. The MRL/lpr mice with little proteinuria and all (4) young MRL/lpr mice glomeruli looked normal under light microscope.

Seven MRL/lpr mice with heavy proteinuria showed changes typical of mild diffuse proliferative lupus nephritis characterized by glomeruli that show diffuse, mild or moderate, global mesangial expansion by excess cells and matrix. The glomeruli in figure 3.1b show moderate, diffuse proliferative changes with increased cellularity, fibrinoid material and decreased vascularity. Figure 3.3c is an electron micrograph of a portion of a glomerulus that shows increased numbers of cells and matrix.

Thirteen MRL/lpr mice with heavy proteinuria had advanced nephritis characterized by lesions ranging from focal to diffuse endocapillary proliferation with basement membrane thickening, necrosis and/or crescents. Figure 3.1c illustrates some glomeruli that show significant increase in size, complete loss of vascularity, thickening of capillary walls, lobulation of glomerular tufts, fibrinoid necrosis and large cellular capsular crescents. There is increased number of infiltrating cells in the interstitium. Figure 3.1d shows advanced (end-stage) diffuse proliferative GN. This glomerulus shows proliferation, capsular crescent, necrosis indicated by fragmented nuclei and fragmented erythrocytes, sclerosis and hyalinization. A wire-loop like lesion is present on the upper right.

At the ultrastructural level, a prominent feature of immune complex mediated lupus nephritis is the deposition of immune complexes in the mesangial, sub-endothelial and sub-epithelial regions. Figure 3.3d shows a portion of a glomerulus with sub-
endothelial deposits. Figure 3.3e illustrates a portion of a glomerulus with both sub-endothelial and sub-epithelial dense deposits. Figure 3.3f illustrates a region of a glomerulus with an advanced lesion. The lumen of the capillary can only be identified by the presence of an infiltrating cell. The capillary wall is thickened by dense deposits in the sub-endothelium. This may be seen as a "wire-loop-like" lesion under light microscope.

The presence of fibrin in glomeruli was assessed using picro-Mallory stain. Presence of fibrin in glomeruli is indicated by intense pink staining. Glomeruli from normal mice show no fibrin (Figure 3.2a). Four mice with mesangial proliferation show fibrin while three mice do not have any fibrin in their glomeruli (Figure 3.2b). All mice with advanced glomerular disease had fibrin in their glomeruli (figure 3.2c and 3.2d).

3.3.2 Expression of factor X activator

Direct immunofluorescence was performed to localize the expression of factor X activator. Our first aim was to determine if the expression of this factor is restricted to the kidney or whether it is expressed in other organs as well. Our second aim was to see if there was a correlation between the expression of this molecule, the severity of the disease and the deposition of fibrin.

The results from immunohistochemical staining using the FITC - labeled H4 on young and old MRL/lpr mice are presented in Figure 3.4. Staining was present only in the glomeruli of diseased MRL/lpr mice with binding mainly seen in the mesangium and capillary walls (figure 3.4b). Immunofluorescence was negative on all young MRL/lpr mice kidneys (figure 3.4a) and on young and old MRL/lpr mice liver, spleen, heart and lung (data not shown). Furthermore, to eliminate any false staining, fluorescein labeled normal mouse IgM was used to stain kidney sections and we found that the antibody did not bind to anything.
Table 3.1 summarizes the light microscopic observations and the immunofluorescence results. It is seen that in 10 out of 13 mice with focal or diffuse endocapillary proliferation with necrosis and/or crescents and in 1 out of 7 mice with mesangial proliferation, factor X activator expression was detected by immunofluorescence. All 13 MRL/lpr mice with moderate or severe disease and four out of seven mice with milder lesions had fibrin in the glomeruli. The expression of glomerular factor X activator as evident by immunofluorescence staining also correlated well with the presence of functional glomerular PCA as measured in a one stage clotting assay (PCA was measured only for 5 mice).

3.3.3 Ultrastructural localization of factor X activator

Immunogold staining was performed to localize factor X activator at the ultrastructural level to identify the cellular source. The immunogold staining results are presented in Figure 3.5. As a positive control for the technique of immunogold staining, anti-murine actin was used as the primary antibody and NANOGOLD-conjugated goat anti-murine IgG was used as the second antibody. In Figure 3.5a, staining is seen in the brush border of proximal tubules. Actin is present in the brush border of proximal tubules (Figure 3.5a) and in the foot processes of epithelial cells (Figure 3.5b). When the NANOGOLD-conjugated goat anti-murine IgG alone was used to stain glomeruli of diseased MRL/lpr mice, no staining was seen (Figure 3.5c). When glomeruli from young MRL/lpr mice were stained with NANOGOLD-conjugated H4, staining was absent (results not shown). However, when H4 was used to stain glomeruli from diseased MRL/lpr mice expressing factor X activator by immunofluorescence, staining was seen in a number of places. Firstly, staining was abundant in the sub-endothelial dense deposits (Figure 3.5d) and sometimes staining in this region was seen in close proximity to “fibrin-like” material (Figure 3.5e). Secondly, staining was seen in phagosomes of macrophages in the glomeruli (Figure 3.5f). Finally, staining was seen in the endothelium and inside the capillary lumen (Figure 3.5g).
Figure 3.1: Histopathology of the kidney in MRL//pr mice. Haematoxylin and eosin stained sections of renal cortex.

a) **Normal control (young) animal.**
   Overall structure of the renal cortex is shown. Four glomeruli; multiple tubules and vessels are well seen. Glomeruli are of normal size and vascularity. x100

b) **Early lesion of diffuse proliferative GN (old animal with proteinuria).**
The two glomeruli shown are increased in size, show increased cellularity due to proliferation of mesangial and endothelial cells and infiltration with polymorphs. There is also mild focal interstitial inflammation. x350

c) **Moderate diffuse proliferative GN (old animal).**
The three glomeruli seen show increased cellularity, thickening of the mesangium, fibrinoid necrosis, lobulation, decreased vascularity and prominent crescents. Tubules show mild atrophy and there is notable interstitial inflammation. x250

d) **Severe lesion of diffuse proliferative GN (old animal).**
Close-up of glomerulus showing severe changes: extensive fibrinoid necrosis and hyalinization, mesangial thickening and decreased vascularity. The linear eosinophilic material on the upper right is a wire-loop lesion (mouse equivalent). There is also diffuse epithelial proliferation with crescent formation. x500
Figure 3.2: Picro-Mallory stain to demonstrate fibrin (pink staining) in glomerular lesions. Lesions are matched to compare with the lesions shown in Figure 3.1.

a) Normal staining, no fibrin present. x100

b) Mild proliferative GN, negative for fibrin. x350

c) Moderate proliferative GN. The glomeruli present are all positive for fibrin. x250

d) Severe proliferative GN. Very extensive fibrin (fibrinoid necrosis) and deposits in the interstitium are present. x500
Figure 3.3: Transmission electron micrographs

a) **Electron micrograph of part of a normal glomerulus.** Several capillary lumina (L) are present; two contain erythrocytes (E), and in two, the endothelial cells (En) are in the plane of section. Urinary spaces (U) contain many primary processes from visceral epithelial cells, but no cell bodies are seen; foot processes (arrow head) are prominent, and are embedded in the outer layer of the capillary basement membrane (Arrows). Bowman's capsule (BC) lined by a layer of parietal epithelial cells (PE) is evident. (x7 800)

b) **Normal glomerulus.** This magnification of figure 3.3a demonstrates the trilaminar structure of the capillary basement membrane (BM), and the endothelial cell (En) cytoplasm lining the capillary lumen (L) is regularly fenestrated (arrow). Within the urinary spaces (U), primary processes of epithelial cells (Ep) give rise to alternate, interdigitating foot processes embedded in the lamina rara externa of the basement membrane. (17 920) (Arrow heads)

c) **Part of a glomerular segment in proliferative GN.** The mesangial region is expanded by both matrix (M) and cells, some of which are probably monocytes (Mo) and some mesangial cells (Mc). Two capillary lumina (L) can be seen. (x7 800)

d) Glomerular capillary wall of a 5 month old MRL/lpr mouse showing sub-endothelial dense deposits (asterisk). The podocytic foot processes (arrow head) separated by filtration slits appear normal, but the fenestrated endothelial cell cytoplasm is lost. (x25 760)

e) Electron micrograph of a segment of a glomerulus showing both sub-endothelial (asterisk) and sub-epithelial (arrow head) dense deposits. (x32 000)

f) **A glomerular segment of MRL/lpr mouse with severe lupus nephritis.** No capillary lumen can be seen. Its position is identified by an occupying infiltrating cell (C). The foot processes are fused. There is thickening of basement membrane by extensive deposits in the sub-endothelium (asterisk). This would be seen as a "wireloop-like" lesion under light microscope. (x17 920)
Figure 3.3e
Figure 3.4. **Immunohistochemistry.** Normal and diseased MRL/lpr mice kidney sections were stained with FITC labeled H4. (a) Kidney cortex from a young MRL/lpr mouse showing no staining. (b) Kidney cortex from old MRL/lpr mouse with lupus nephritis showing staining in the mesangium and capillary wall.
**Figure 3.5:**

a) Silver enhanced immunogold labeling of normal young MRL/lpr mouse kidney cortex with an indirect conjugate of murine anti-actin antibody followed by a goat anti-murine IgG antibody. Silver enhanced gold particles are seen in the brush border of proximal tubules (arrow heads). Bar = 500 nm.

b) Silver enhanced immunogold labeling of a diseased MRL/lpr mouse kidney cortex with an indirect conjugate of murine anti-actin antibody followed by a goat anti-murine IgG antibody. Silver enhanced gold particles are seen in the podocytes' foot processes (arrow heads). Bar = 200 nm.

c) Silver enhanced immunogold labeling of diseased MRL/lpr mouse glomerulus with goat anti-murine IgG. No silver enhanced gold particles were detected in the dense deposits (asterisk), basement membrane (arrow heads) or erythrocytes (E). Bar = 0.5 μm.

d) Silver enhanced immunogold labeling of diseased MRL/lpr mouse glomerulus with a direct conjugate of H4. Sub-endothelial deposits (asterisk) are heavily decorated with silver enhanced gold particles. Bar = 0.5 μm.

e) Silver enhanced immunogold labeling of diseased MRL/lpr glomerulus with a direct conjugate of H4. Sub-endothelial deposits are heavily decorated with silver enhanced gold particles (asterisk). Note the "fibrin-like" deposits (arrow heads) in association with the labeled material. Bar = 500 nm.
f) Silver enhanced immunogold labeling of diseased MRL/lpr glomerulus with direct conjugate of H4. Macrophages found in the mesangium contained phagosomes which were heavily decorated with silver enhanced gold particles (arrow heads). Sub-endothelial deposits seen are also labeled (asterisk). Bar = 2 μm.

g) Silver enhanced immunogold labeling of diseased MRL/lpr glomerulus with direct conjugate of H4. The capillary lumen contains platelet (P) in association with serum proteins (arrow heads). Silver enhanced gold particles were detected in the serum proteins as well as the endothelium lining the lumen (arrows). Bar = 500 nm.
Figure 3.5f
Figure 3.5g
Table 3.1

Summary of light microscopic observations

<table>
<thead>
<tr>
<th>Glomerular morphology</th>
<th>Number of mice</th>
<th>Number of mice with fibrin</th>
<th>Number 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 proliferation (++)</td>
<td>13</td>
<td>13</td>
<td>10</td>
</tr>
</tbody>
</table>

H&E staining was performed on young MRL/lpr mice (normal) and old MRL/lpr mice (with proteinuria) and the extent of glomerular injury was noted. Picro mallory stain was used on these mice to assess the presence of fibrin. The presence of factor X activator was detected by direct immunofluorescence using the H4 monoclonal antibody.
CHAPTER 4: DISCUSSION

The association between glomerular damage in GN and activation of the coagulation system has been known for some time. The importance of activation of coagulation as a mediator of glomerular damage has been emphasized by studies showing that defibrination reduced the incidence of glomerular microthrombi (160), reduced both fibrin deposition in the glomeruli and crescent formation (161), protected against experimental nephrotoxic serum nephritis (119), delayed the development of renal fibrin deposition and GN, improved survival in BXSB male mice (121) and improved renal function and reduced glomerular thrombi in patients with lupus nephritis (120).

We have previously shown that peripheral blood monocytes/splenic macrophages from humans and mice with active lupus nephritis express large amounts of PCA (153, 154). We chose to study MRL/lpr mouse model in our studies because of the similarity between the mouse model and the human disease. Most of the pathology seen in these mice are identical to that of lupus patients. The present studies were undertaken to quantify and characterize 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 was due to a factor X activator because for the full expression of this activity only coagulation factors X, V and prothrombin were needed. Further support for this finding came through our observation that the glomerular lysate from the MRL/lpr mice directly activated factor X. No activation of prothrombin occurred in the absence of factor X, thereby excluding the presence of a direct prothrombinase activity which our lab has identified with a model of viral hepatitis (145).

Our studies indicate that there is a unique factor X activator in the glomeruli of MRL/lpr mice with lupus nephritis. Factor X is the proenzyme of the Vitamin K-
dependent serine protease factor Xa. Normally, activation of factor X is accomplished through the incorporation of factor X into either of the two membrane bound complexes: the factor IXa-VIIIa complex and the tissue factor-VIIa complex as evident in figure 1.2. The possibility of glomerular factor X activator being the factor IXa-VIIIa complex was ruled out because for the expression of this activity, none of the intrinsic coagulation factors including factors IX and VIII, were needed. The second possibility of glomerular factor X activator being tissue factor was excluded due to a number of our observations. First of all, glomerular PCA did not require factor VII for its expression. Even though it can 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 (128,162), our next set of observations provide evidence against it. The size of factor X activator in a non-reduced gel was determined to be approximately 66 kD, whereas the size of tissue factor is about 31 kD for the non-glycosylated form and about 46 kD for the glycosylated form. As well, H4 (a monoclonal antibody to glomerular PCA) did not recognize any protein in LPS stimulated macrophages which was expressing tissue factor. Next, H4 specifically inhibited glomerular factor X activation but not tissue factor. It would have been ideal to determine whether an antibody against tissue factor inhibited glomerular PCA. However, a functionally neutralizing antibody to murine tissue factor was not available. Finally, Con A, at doses that substantially inhibited tissue factor, did not inhibit glomerular factor X activator.

To further characterize glomerular factor X activator, the effect of several protease inhibitors on glomerular PCA was studied. Glomerular factor X activator was identified to be serine protease because it was significantly inhibited by serine protease inhibitors PMSF and aprotinin, but not by E-64, a cysteine protease inhibitor. Because most coagulation proteases including factors Xa and thrombin are serine proteases, one needs to be careful in making a decision about the nature of the protease from a one stage clotting assay. Because PMSF inhibits most of the serine proteases involved in coagulation, the inhibition observed with PMSF could have been due to its inhibitory effect on factor Xa and thrombin. Aprotinin however, has no inhibitory effect on factor
Xa nor thrombin, thereby confirming that factor X activator is a serine protease.

We then attempted to localize the expression of glomerular factor X activator and to assess the association between the extent of glomerular damage, fibrin deposition and the expression of factor X activator in diseased glomeruli. Immunofluorescence studies using H4 revealed that factor X activator expression was restricted to the glomeruli of diseased MRL/lpr mice with staining seen in the mesangium and the capillary wall. Expression of factor X activator was observed in a majority of old MRL/lpr mice with focal or diffuse proliferative lesions and fibrin deposition in the glomerular tuft. As further support for the association of glomerular factor X activator expression and fibrin, we detected factor X activator in close proximity to fibrin-like structures in the sub-endothelial deposits. In normal glomeruli, on the other hand, neither factor X activator nor fibrin was detected. The amount of functional glomerular factor X activator (clotting assay) correlated with the presence of factor X activator as indicated by immunoblot and by immunofluorescence (data not shown).

Factor X activator expression was localized at an ultrastructural level to the dense deposits in the mesangial and sub-endothelial regions. In some glomeruli, factor X activator was also seen in macrophages, both in phagosomes and cytoplasm, and altered or activated endothelial-like cells. Factor X activator could be synthesized elsewhere, and get transported to the kidney via the blood, or it could be produced locally by cells in the glomeruli (either macrophages or endothelial cells). In support of the first possibility we detected factor X activator in the dense deposits at the sub-endothelial and mesangial regions, the sites which have access to the systemic circulation. We also detected factor X activator in the lumen of a capillary associated with some serum components and in phagosomes of macrophages which could have been from phagocytosis of deposits by macrophages. Kanno et al. demonstrated that macrophages can phagocytose materials deposited in glomerular sub-endothelial regions and mesangium (163). However, there are a number of reasons why it is unlikely to be synthesized elsewhere and transported to the kidney. First of all, we did not detect the presence of this protein in any other organs.
including liver, spleen, lung and heart. Secondly, macrophages are characteristically the source of procoagulants; however, we did not observe the expression of factor X activator in macrophages from other tissues including spleen, lung, liver and heart. The presence of protein in the capillary with serum could have resulted through the lysis of cells and the release of factor X activator into the capillary.

The second possibility is that glomerular factor X activator is produced locally by endothelial cells and/or macrophages. Both cell types are capable of producing procoagulants in response to immune complexes, tumor necrosis factor (TNF), interleukin 1 (IL-1), thrombin, endotoxin, C5a, and mechanical injury (164,165,166,137,167,168,169,131,145,170). In MRL/lpr mice with lupus nephritis, TNF-α and IL-1β are upregulated (171) and these cytokines could trigger the expression of factor X activator in the glomeruli by either the endothelial cells or the macrophages.

Support for the endothelial cell as the cellular source of factor X activator comes from a number of observations made by others. It had been shown by Haviland et al that human umbilical vein endothelial cells (HUVECs) have receptors to C5a (172). Recently, Ikeda et al. have shown that HUVECs can produce significant amounts of procoagulants (tissue factor) in response to C5a (165). Tannenbaum et al. had reported that immunoglobulins and immune complexes from the sera of patients with lupus can induce PCA in human umbilical endothelial cells (166). So it is possible that glomerular endothelial cells produce factor X activator in response to cytokines, complement or immune complexes.

Accumulation of macrophages is a prominent feature in many forms of human and experimental GN (173,174). Accumulation of macrophages in MRL/lpr mice kidneys is particularly notable and the increase in the number of macrophages before the onset of disease suggested that macrophages may be involved in promoting renal injury (175). This is further supported by the observation that colony-stimulating factor-1 (CSF-1 which can regulate the survival, proliferation and differentiation of macrophages) can
initiate lupus nephritis (175-177). Even though, there is compelling evidence to implicate macrophages in the development of lupus nephritis, the mechanism by which they contribute to disease has not been clearly dissected. One possible mechanism by which macrophages of the kidney contribute to the development of nephritis could be through the production of glomerular factor X activator. In many experimental models of GN, the increase in PCA occurs in parallel to the accumulation of glomerular macrophages and precedes the deposition of fibrin within the glomeruli. In acute serum sickness nephritis, anti-GBM GN, a passive model of autologous-phase GN, crescentic GN in rabbits and human proliferative GN (127), the accumulation of macrophages is prominent and glomerular PCA is significantly increased (127,128,131). The relatively small amount of PCA in resting macrophages increases significantly in response to immune complexes (137,167,168), C5a (169), endotoxin (131), and viruses (145). Therefore, it is possible that in MRL/lpr mice, macrophages accumulate in the kidney and produce large amounts of factor X activator in response to one or more of the above mentioned stimuli.

A number of factor X activators have been described to date. 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 by Francis and Colleagues (178,179). This conclusion was based on enhancement of PCA by the addition of purified factor VII and inhibition by an antibody to factor VII. The lack of dependence of glomerular PCA on factor VII and the selective inhibition of glomerular PCA but not tissue factor by H4 suggest that the glomerular factor X activator is different from that described above. Shands has described a factor X activator in mouse peritoneal exudate macrophages which also appears to be a complex of membrane-bound tissue factor and factor VII - like substance, the factor VII produced by the macrophages themselves (144). Gordon and co-workers have identified a factor X activator in extracts of human and animal tumors and in cultured malignant cells (143). However, this PCA was found to be a cysteine protease. More recently, the production of a factor X activator by a methylcholanthrene - induced rat fibrosarcoma has also been described (180). This activity was similar to glomerular PCA in terms of its relative resistance to Con A and E-
64 and sensitivity to inhibition by aprotinin. However, the fibrosarcoma-associated factor X activator was not inhibited by PMSF and it was not dependent on calcium for activity, whereas the glomerular factor X activator described in this study appears to be dependent on calcium for its functional activity and it is inhibited by PMSF.

Wiggins and colleagues (128) as well as Holdsworth and Tipping (130) have shown that PCA is induced in glomerular macrophages from rabbits with anti-glomerular basement membrane antibody mediated GN. Studies by Hancock and colleagues, which utilized a monoclonal antibody directed against macrophage activation antigen, showed mononuclear cell surface expression of PCA in patients with active GN (181). In anti-glomerular basement membrane antibody-induced GN in rabbits, induction of macrophage PCA preceded fibrin deposition and it was suggested that PCA deposition was responsible for fibrin accumulation in this disease (129). In both nephrotoxic nephritis and acute serum sickness, glomerular fibrin accumulation was associated with augmented glomerular PCA which had the characteristics of tissue factor (162,182). Glomerular macrophages were strongly implicated as the cell most responsible for elaboration of PCA. A study in two patients with crescentic GN also concluded that the increased glomerular PCA seen in these patients was tissue factor (127). Therefore, our study is the first study describing a factor X activator that is distinct from tissue factor in the glomeruli.

The mechanism by which factor X activator contributes to glomerular disease is uncertain. Factor X activator can activate factor X and lead to the production of thrombin. Furthermore, activated factor X (factor Xa) can induce enhanced release of endothelial cell mitogens such as platelet-derived growth factor like molecules (183). Thrombin is a chemoattractant for monocytes and platelets and it stimulates mononuclear cell binding to endothelial cells (184,185). In addition thrombin can also convert fibrinogen to fibrin which could cause tissue damage by interfering with the microcirculation, and by reducing macrophage mobility. Fibrin degradation products can be cytotoxic to mesangial cells and attract circulating inflammatory cells (118,186,187).
Glomerular factor X activator could cause injury by one or more of these mechanisms or via a novel mechanism.

How does one relate the expression of glomerular factor X activator to lupus nephritis in MRL/lpr mice? The most likely explanation is that as a result of defective expression of fas receptor, autoreactive T cells escape the selection process and lead to the formation of autoantibodies. Immune complexes consisting of autoantibodies and their antigen, deposit in the kidney and induce macrophages and/or endothelial cells to produce factor X activator. A number of observations provide evidence for the notion that immune complexes can induce expression of PCA. Our laboratory has previously reported that circulating procoagulant inducing factor (PIF), possibly immune complexes, in the serum of BXSB male mice induced the lymphocytes to stimulate the synthesis of PCA by splenic macrophages (154,188). Schwartz et al. demonstrated that immune complexes can induce increased production of PCA by human monocytes (137). Finally, Tannenbaum et al. demonstrated that sera from SLE patients containing immune complexes induced PCA in human umbilical endothelial cells (166). The fact that factor X activator is only seen in the kidney may reflect differential production of factor X activator by resident cells of the kidney due to the presence of large amounts of stimulatory molecules inducting factor X activator or the absence of inhibitory cells or molecules that are present in other organs. Favoring this is the observation that glomerular endothelial cells in MRL/lpr mice with lupus nephritis synthesize increased amounts of type 1 plasminogen activator inhibitor (PAI-1) in response to TNF-α and IL-1β (189). Interestingly PAI-1 inhibits the conversion of plasminogen to plasmin which might contribute to the persistence of glomerular fibrin deposits in mice with GN.

Thus, these studies describe a unique factor X activator in murine glomeruli associated with the development of proliferative lupus nephritis. The expression of this molecule is restricted to the glomeruli and it correlates well with the extent of glomerular damage and the presence of fibrin.
Future studies

In order to identify the cellular source of factor X activator, a detailed immunoelectron microscopic study needs to be undertaken. Another approach that can be taken in search of an answer to that question and many other questions is to isolate the gene that encodes for this protein. Isolating the gene can be accomplished in a number of ways. Since a monoclonal antibody against factor X activator is available, an expression cDNA library constructed from the kidney cortex of mice expressing high levels of factor X activator could be screened to isolate the cDNA encoding factor X activator. A second method using differential display could be employed to identify the gene. Differential display can be performed by comparing the mRNA species present in diseased MRL/lpr mice expressing high levels of factor X activator and young MRL/lpr mice without factor X activator expression. A third method that can also be used to isolate the gene would be to perform protein micro-sequencing. For this purpose, isoelectrofocusing gel electrophoresis could be performed and the protein band corresponding to the size of factor X activator obtained in an immunoblot, excised, eluted and micro-sequenced.

Once the gene is isolated, in situ hybridization could be carried out to localize the cellular source of the mRNA which will identify the source of the protein. Furthermore, studies could then be undertaken to determine the mechanism of regulation of the gene. Two approaches which could be undertaken would be the production of mutation deletion mice (knock out) to study the implication of under-expression of the gene and transgenic mice to study the implication of over-expression of factor X activator.

All of the above studies will clearly identify the importance of factor X activator in murine lupus nephritis.
CHAPTER 5: REFERENCES


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