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Fibrin independent proinflammatory effects of tissue factor in experimental crescentic glomerulonephritis

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Background. Tissue factor initiated glomerular fibrin deposition is an important mediator of injury in crescentic glomerulonephritis. Recent data have suggested noncoagulant roles for tissue factor in inflammation.

Methods. To test the hypothesis that in addition to its effects in initiating coagulation, tissue factor has proinflammatory effects in glomerulonephritis, rabbits given crescentic anti-glomerular basement membrane (GBM) antibody–induced glomerulonephritis were defibrinogenated with ancrod. One group of defibrinogenated rabbits was also given anti-tissue factor antibodies. Comparisons were made between these groups, as well as a third group that was neither defibrinogenated with ancrod nor given anti-tissue factor antibodies.

Results. Defibrinogenation alone abolished glomerular fibrin deposition, reduced crescent formation, and limited renal impairment (ancrod-treated, serum creatinine 274 ± 37 μmol/L; untreated 415 ± 51 μmol/L; $P < 0.01$). Tissue factor inhibition in defibrinogenated rabbits resulted in further protection of renal function (creatinine 140 ± 19 μmol/L, $P < 0.01$) and reduced proteinuria (0.4 ± 0.2g/day, untreated 2.6 ± 0.4 g/day, $P < 0.01$), which was significantly increased by defibrinogenation alone (ancrod-treated, 5.6 ± 1.2 g/day). Anti-tissue factor antibodies (but not defibrinogenation alone) attenuated glomerular T-cell and macrophage recruitment, and major histocompatibility complex (MHC) class II expression.

Conclusion. These results demonstrate important proinflammatory effects of tissue factor in crescentic glomerulonephritis that are fibrin independent and provide in vivo evidence for tissue factor's proinflammatory effects on MHC class II expression and leukocyte accumulation.

In human crescentic glomerulonephritis, rapid and often irreversible loss of renal function is strongly associated with prominent glomerular fibrin deposition [1–4]. The association of glomerular fibrin deposition with an influx of T cells and macrophages together with up-regulation of tissue factor expression in crescentic glomerulonephritis suggests fibrin deposition may occur as part of an immune delayed-type hypersensitivity response [3, 4]. Studies using ancrod, a snake venom extract which converts fibrinogen to a soluble fibrin monomer which is rapidly depleted from the circulation, have demonstrated that fibrin is an important mediator of injury in human [5] and experimental [6–9] crescentic glomerulonephritis.

Tissue factor initiates the extrinsic coagulation pathway by binding circulating factor VII and facilitating its allosteric conversion to factor VIIa [10–12]. Recent studies suggests that tissue factor may also contribute directly to the inflammatory injury in crescentic glomerulonephritis by augmenting macrophage activation and T-cell proliferation, effects which appear to be mediated through its intracytoplasmic tail [13]. The important role of tissue factor in glomerular coagulation and crescent formation has recently been confirmed by experimental studies showing that specific inhibition of tissue factor functional activity by either antibody inhibition [14] or infusion of the natural biologic inhibitor of tissue factor, tissue factor pathway inhibitor (TFPI) [15], results in significant attenuation of the development of crescentic glomerulonephritis. Inhibition of tissue factor decreased crescent formation, preserved renal function, and reduced proteinuria. In addition, tissue factor inhibition was associated with decreased glomerular inflammation [macrophage influx and major histocompatibility complex (MHC) class II expression]. The confounding problem in this model (and in disseminated intravascular coagulation where tissue factor blockade has also been shown to be protective [16]) is that coagulation and fibrin deposition are also significantly inhibited. It is therefore possible that the beneficial effects seen were entirely due to inhibition of the known procoagulant effects of tissue factor, thus preventing the injurious fibrin deposition and the generation of factors such as factor Xa and thrombin which have demonstrated proinflammatory effects [17–19].

Key words: tissue factor, crescentic glomerulonephritis, inflammation, macrophage, ancrod.

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Fibrin deposition is an end result of pathologic coagulation. Fibrin is strongly associated with experimental crescentic glomerulonephritis [20, 21], is deposited early in, and is critical to disease progression [8]. Experimentally, defibrinogenation with ancrod from the commencement of injury [6–8] or the prevention of further fibrin deposition or removal of deposited fibrin (with ancrod or streptokinase) in established disease [9] decreased impairment of renal filtration and crescent formation. However, many parameters of cellular inflammation were unaffected and proteinuria was significantly exacerbated. There was no reduction in glomerular influx of macrophages. Taken together, these data confirm a key role for pathologic fibrin deposition in the formation of glomerular crescents and the development of renal failure due to reduction in glomerular filtration. However, preservation of filtration and continued macrophage-associated glomerular inflammation lead to increased pathologic proteinuria and continued cellular proliferation. These ongoing pathologic events may be contributed to by the continued proinflammatory effects of augmented tissue factor activity in the absence of fibrin deposition.

Previously various anticoagulant strategies in human crescentic glomerulonephritis have been studied, notably heparin, warfarin, and ancrod [5]. Despite initial enthusiasm, the clinical benefits in human disease have overall been disappointing [22, 23]. Tissue factor is best known for its procoagulant role but its proinflammatory effects are increasingly recognized [16, 24]. Inhibition of tissue factor may have particular benefits in situations where both inflammation and pathologic fibrin deposition contribute to injury as in crescentic glomerulonephritis. Thus it is important to dissect the relative procoagulant from proinflammatory effects of tissue factor in crescentic glomerulonephritis. In order to study the role of tissue factor receptor–mediated inflammatory effects independent of procoagulant/fibrin-mediated effects, the current study assessed the effects of the inhibition of tissue factor in rabbits developing crescentic anti-glomerular basement membrane (GBM) glomerulonephritis simultaneously defibrinogenated by the use of ancrod.

METHODS

Experimental design

Experimental accelerated autologous anti-GBM antibody initiated glomerulonephritis was induced in male New Zealand White rabbits, 1.9 to 2.3 kg body weight (Monash University Central Animal Services, Clayton, Victoria, Australia). Infusions were given via plastic cannulas inserted into the right external jugular vein under (ketamine/xylazine) anesthetic. Rabbits were bled daily via the catheters to allow estimation of serum creatinine, the clot quality test and fibrinogen levels. Autologous phase anti-GBM glomerulonephritis was initiated by the injection of horse antirabbit GBM serum as a single intravenous bolus dose of 25 mg/kg at day 0 to rabbits sensitized by subcutaneous injection 5 days previously with 4 mg of normal horse globulin in 1 mL Freund's complete adjuvant (Sigma, Castle Hill, NSW, Australia). The following groups of rabbits with glomerulonephritis were studied: (*1*) control treatment ($N = 5$) on rabbits with glomerulonephritis that received saline (vehicle for ancrod) and normal sheep globulin (control for anti-tissue factor antibodies); (*2*) ancrod treatment alone ($N = 5$) on rabbits with glomerulonephritis that were defibrinogenated with ancrod and also received normal sheep globulin (control for anti-tissue factor antibodies); and (*3*) ancrod treatment and anti-tissue factor antibodies treatment ($N = 5$) on rabbits with glomerulonephritis that were both defibrinogenated with ancrod and also received anti-tissue factor antibodies.

Twenty-four hours prior to the injection of anti-GBM globulin, sensitized rabbits in groups 2 and 3 were treated intravenously with ancrod (Knoll, Ludigshafen, Germany) dissolved immediately before use (5 mL 0.9% saline). The initial dose of 0.5 U/kg (over 60 minutes) was followed immediately by a 2-hour infusion of 1.5 U/kg. Further doses were given 12 hourly (2 U/kg) as a slow intravenous infusion. The dose of ancrod was adjusted daily when required to keep the clot quality test of Reid, Chan, and Thean [25] at 0 or 1. Sheep antirabbit tissue factor antibodies were prepared according to a previously published method [14]. Daily intravenous bolus injections of functionally inhibitory sheep antirabbit tissue factor antibodies (50 mg/kg) were commenced 2 hours after the induction of glomerulonephritis (group 2). Rabbits in groups 1 and 3 received normal sheep globulin, 50 mg/kg intravenously daily. Four days after initiation of anti-GBM glomerulonephritis rabbits were sacrificed. At the time of sacrifice, no rabbit displayed any evidence of internal or external hemorrhage.

Routine hematologic parameters of platelet and white blood cell count and hemoglobin concentrations were assessed on blood [ethylenediaminetetraacetic acid (EDTA) as anticoagulant] on a Coulter STKS analyzer (Coulter Electronics, Miami, FL, USA). Measurements of the activated partial thromboplastin time (aPTT), prothrombin time (PT), and plasma fibrinogen concentrations were performed on citrated blood specimens using an Electra 1000C automatic coagulation time apparatus (Medical Laboratory Automation Inc., Pleasantville, NY, USA). The clot quality test of Reid, Chan, and Thean [25] was performed once daily on nondiluted blood aspirated from the jugular line. Results are expressed as the mean ± SEM and the significance of differences between groups were analyzed by analysis of variance (ANOVA), followed by the Neuman-Keuls post hoc test.

Table 1. Blood counts and coagulation parameters in rabbits used in these studies

	Normal	Control glomerulonephritis	Ancrod	Ancrod and anti-tissue factor
Hemoglobin *g/L*	12.3 ± 1.8	11.6 ± 2.4	12.8 ± 2.7	11.5 ± 2.1
White cell count × 10^9/*L*	5.6 ± 1.2	6.4 ± 2.6	7.1 ± 1.4	5.9 ± 0.9
Platelets × 10^9/*L*	320 ± 21	402 ± 78	289 ± 80	334 ± 43
Fibrinogen μ*mol/L*	2.23 ± 0.40	2.96 ± 0.39	0.20 ± 0.13	0.16 ± 0.10
aPTT *seconds*	16.8 ± 1.8	19.5 ± 1.4	NCD	NCD
PT *seconds*	0.9 ± 0.1	0.9 ± 0.2	NCD	NCD

Abbreviations: aPTT, activated partial thromboplastin time; PT, prothrombin time; NCD, no clot detected.
Ancrod affected coagulation parameters, including fibrinogen levels, the aPTT and PT, but not the hemoglobin concentration, white cell count, or platelet count. The addition of anti-tissue factor antibodies to ancrod-treated rabbits had no additional effects on coagulation.

Assessment of glomerular injury

Kidney tissue was fixed in Bouin's fixative, embedded in paraffin, and 2 μm tissue sections were cut and stained with periodic acid-Schiff (PAS) reagent. A minimum of 50 glomeruli was assessed to determine the crescent score for each animal. The extent of crescent formation in each glomerulus was scored semiquantitatively (0 to 3+) as follows: 0, no crescent present; 1, crescent occupying up to one third of Bowman's space circumferentially; 2, crescent occupying one third to two thirds of Bowman's space; and 3, crescent involving greater than two thirds of the circumference of Bowmans space.

Plasma creatinine measurements were performed on plasma samples using the alkaline picric acid method. Proteinuria was calculated after collecting urine over the final 24 hours of each experiment. Rabbits were housed in metabolic cages with free access to fresh water and standard chow. Residual bladder urine was aspirated after sacrifice. Protein concentrations were determined by the Coomassie brilliant blue dye binding assay [26].

Assessment of glomerular fibrin deposition, leukocyte recruitment, and MHC class II

For glomerular fibrin deposition, snap-frozen tissue was embedded immediately in Optimal Cutting Temperature Compound (Miles Scientific, Naperville, IL, USA) and stored at −70°C before 6 μm cryostat cut sections were examined. Fibrin immunofluorescence staining was performed on acetone fixed section (4°C for 2 minutes) followed by incubation with 10% normal goat or sheep serum [5% phosphate-buffered saline (PBS) for 10 minutes, room temperature], then incubated with fluorescein isothiocyanate (FITC)-conjugated sheep antirabbit fibrinogen (Research Plus, Bayonne, NJ, USA) (1:100) for 45 minutes. Slides were washed with PBS, mounted with 50% glycerol, and examined under a fluorescence microscope (Leitz, Oberkochen, Germany). Glomerular fibrin deposition was assessed using a blinded protocol in at least 50 glomeruli according to a previously published protocol [9, 14, 21]. The degree of glomerular fibrin deposition was scored semiquantitatively (0 to 3+) as follows: 0, no fibrin deposition; 1, fibrin occupying up to one third of the glomerular cross-sectional area; 2, fibrin occupying one third to two thirds of the glomerulus; and 3, greater than two thirds of the glomerular cross-section covered by fibrin.

For assessment of leukocytes and MHC class II, kidney and spleen from each animal was fixed in periodate lysine paraformaldehyde for 4 hours, washed (7% sucrose in PBS), then frozen in liquid nitrogen-cooled isopentane. Four micrometer sections were stained with either Ken 5 (anti-CD5, a pan T-cell marker) [27], RAM 11 (macrophages) [28], or 2CAB12 (rabbit MHC class II antigen) [29], followed by rabbit antimouse IgG globulin (Dako, Glostrup, Denmark) (1:100), then horseradish peroxidase and mouse antihorseradish peroxidase immunoglobulin (Dako) (1:100). Sections were incubated with diaminobenzadine (Sigma), and counterstained with Harris hematoxylin. Sections of spleen provided a positive control for each animal and either an irrelevant isotype-matched monoclonal antibody or protein G-purified mouse IgG was substituted for the specific monoclonal antibody to provide a negative control. Glomerular leukocyte cell numbers were assessed using a blinded protocol. A minimum of 50 equatorially sectioned glomeruli per animal were assessed per animal and the results were expressed as cells per glomerular cross-section (c/gcs). Expression of MHC class II in glomeruli was assessed semiquantitatively in a blinded protocol by grading the staining from 0 to +4 according to a previously published protocol [30]. Glomeruli with no staining scored 0; 1, up to 10% positive; 2, 10% to one third positive; 3, one third to two thirds; and 4, greater than two thirds positive.

RESULTS

Defibrinogenation with ancrod prevents pathologic glomerular fibrin deposition in crescentic glomerulonephritis

Serum fibrinogen levels were 2.96 ± 0.39 μmol/L in unmodified, control treated disease but were rarely within the detectable range in the two ancrod-treated groups (ancrod-treated alone 0.20 ± 0.13 μmol/L; ancrod and anti-tissue factor-treated 0.16 ± 0.10 μmol/L) (Table 1). In

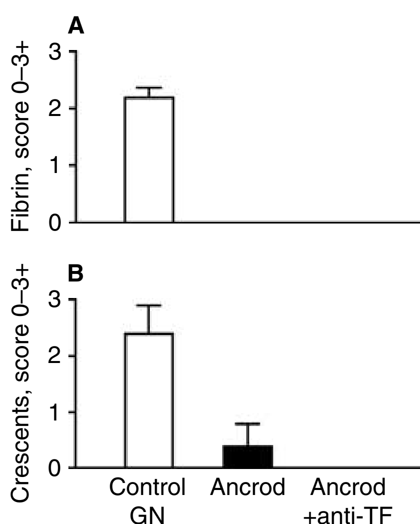

Fig. 1. Glomerular fibrin deposition and glomerular crescent formation in control-treated rabbits with glomerulonephritis (GN), rabbits with glomerulonephritis given ancrod alone, and rabbits given ancrod and anti-tissue factor (TF) antibodies. (*A*) Significant and extensive glomerular fibrin deposition was observed in rabbits developing experimental crescentic anti-glomerular basement membrane (GBM) glomerulonephritis. Ancrod treatment abrogated this pathologic glomerular fibrin deposition. (*B*) Associated with glomerular fibrin deposition extensive crescent formulation characterizes this model of glomerulonephritis. This was significantly attenuated by ancrod-induced defibrinogenation, although a minor component of crescent formation was refractory to treatment. Combined ancrod and anti-tissue factor antibody treatment abrogated glomerular crescent formation.

keeping with the degree of defibrinogenation, there was no clot detected in either of the ancrod-treated groups. In contrast, the aPTT in the control treated disease group was 19.5 ± 1.4 seconds (normal, no glomerulonephritis 16.8 ± 1.8 seconds). The full blood examination was not affected by ancrod or combined ancrod and anti-tissue factor treatment. In particular, platelet counts were not significantly different between the three groups of rabbits with glomerulonephritis. Hemoglobin, total leukocyte count, and circulating monocyte numbers were similarly unaffected. Significant and extensive glomerular fibrin deposition was observed in rabbits with given control treatments [score (0 to 3+) 2.2 ± 0.2] (Fig. 1A), but glomerular fibrin deposition was undetectable in defibrinogenated rabbits with glomerulonephritis (score 0 in rabbits treated with ancrod alone, 0 in those treated with ancrod and anti-tissue factor) (Fig. 1A), indicating complete prevention of glomerular fibrin deposition by this degree of ancrod-induced defibrinogenation.

Effects of defibrinogenation alone or with tissue factor inhibition on renal injury

Frequent and severe crescent formation was seen in control treated rabbits with glomerulonephritis [score (0 to 3+) 2.4 ± 0.5 in unmodified, control-treated glomerulonephritis] (Figs. 1B and 2A and D). As expected, crescent formation was markedly attenuated in rabbits treated with ancrod alone (score 0.4 ± 0.4) (Figs. 1B and 2B and E) and abrogated by the additional treatment of anti-tissue factor antibodies with ancrod (score 0) (Figs. 1B and 2C and F). At the end of experiments (day 4 post-disease induction), control-treated rabbits developed significant renal impairment (415 ± 51 μmol/L) (Fig. 3A). The serum creatinine was 274 ± 37 μmol/L in rabbits treated with ancrod alone ($P = 0.02$ control-treated vs. ancrod-treated). This demonstrates that the known preservation of glomerular filtration by ancrod-induced defibrinogenation was reproduced in this experiment. Additional treatment with anti-tissue factor antibodies (as well as ancrod) further improved renal function (serum creatinine 140 ± 19 μmol/L, $P < 0.005$ control-treated vs. ancrod-treated + anti-tissue factor antibodies, $P = 0.03$ ancrod-treated vs. ancrod-treated + anti-tissue factor antibodies). Thus, although ancrod treatment prevented glomerular fibrin deposition and preserved glomerular filtration, inhibition of tissue factor in addition to defibrinogenation further protected from loss of renal function. Twenty-four–hour urinary protein excretion was 2.6 ± 0.4 g/day in control treated disease (Fig. 3B), 5.6 ± 1.2 g/day in ancrod-treated, and fell to 0.4 ± 0.2g/day in ancrod-treated and anti-tissue factor–treated animals ($P < 0.001$ control-treated vs. ancrod-treated, $P < 0.001$ control-treated vs. ancrod-treated + anti-tissue factor antibodies, and $P < 0.0001$ ancrod-treated vs. ancrod-treated + anti-tissue factor antibodies).

Effects of defibrinogenation with or without anti-tissue factor antibodies on glomerular leukocyte accumulation and MHC class II expression

The monoclonal antibody Ken 5 that detects CD5+ T cells revealed 6.4 ± 1.3 c/gcs in rabbits treated with ancrod alone (Fig. 4A), 2.2 ± 0.7 c/gcs in those treated with ancrod and anti-tissue factor, and 5.8 ± 1.8 c/gcs in control-treated glomerulonephritis ($P < 0.001$ vs. either the control-treated or ancrod-treated groups). RAM 11 antibody staining revealed a glomerular macrophage count of 11.8 ± 2.8 c/gcs (Figs. 4B and 5A) in rabbits with control-treated disease, 12.4 ± 3.0 c/gcs in ancrod-treated rabbits (Fig. 5B), and 4.2 ± 1.9 c/gcs in rabbits treated with both ancrod and anti-tissue factor antibodies ($P < 0.001$ vs. either the control-treated or ancrod-treated groups) (Fig. 5C). MHC class II expression, determined by immunostaining with the monoclonal antibody 2CAB12, was on average, moderate [score (0 to 4) 2.7 ± 0.2] (Fig. 5D and G) in control-treated glomerulonephritis, reduced (2.1 ± 0.2) (Fig. 5E and G) in rabbits treated with ancrod alone and further reduced to 1.6 ± 0.2 (Fig. 5F and G) in rabbits treated with ancrod and anti-tissue factor ($P < 0.005$ for all group comparisons). While

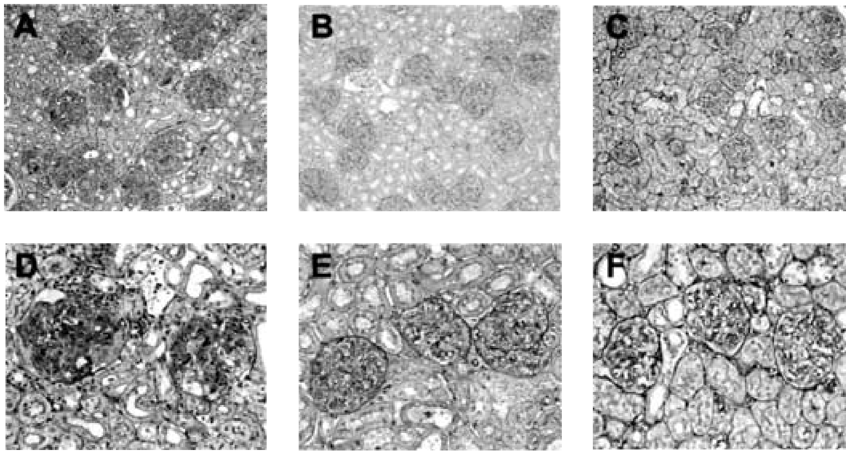

Fig. 2. Histologic injury in rabbits treated with ancrod and ancrod + anti-tissue factor antibodies. Control-treated rabbits with glomerulonephritis developed proliferative glomerulonephritis (*A*) with severe glomerular injury (*D*). Ancrod treatment significantly improved histologic appearances and glomerular injury, but proliferative disease remained (*B* and *E*). Combined ancrod and anti-tissue factor antibody treatment resulted in a further improvement in renal histology (*C* and *F*) compared with ancrod alone [periodic acid-Schiff (PAS) stained, original magnification ×100 (A to C, low power views), ×200 (D to F, high power views)].

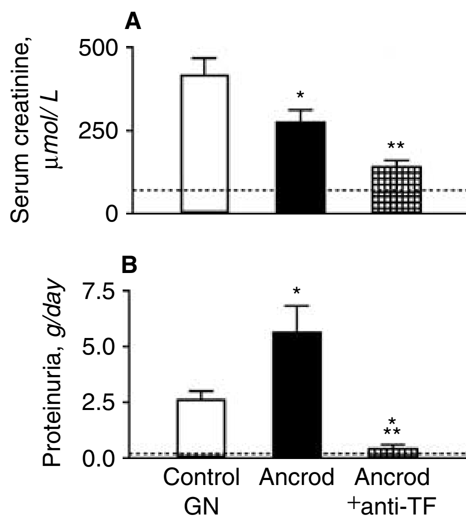

Fig. 3. Functional indices of renal disease in control treated rabbits with glomerulonephritis (GN), rabbits with glomerulonephritis given ancrod alone, and rabbits given ancrod and anti-tissue factor (TF) antibodies. (*A*) Untreated rabbits developing crescentic glomerulonephritis developed severe renal failure manifest by elevated creatinine levels. Ancrod-induced defibrinogenation significantly attenuated renal failure and the addition of anti-tissue factor globulin afforded further significant protection against renal failure. (*B*) Heavy proteinuria developed in untreated animals with crescentic glomerulonephritis that was exacerbated by ancrod-induced defibrinogenation but significantly improved by the addition of anti-tissue factor globulin treatment. Dotted lines represent normal values. (A) $^{*}P = 0.02$ vs. control glomerulonephritis; $^{**}P < 0.005$ vs. control glomerulonephritis, and $P = 0.03$ vs. ancrod treatment alone. (B) $^{*}P < 0.001$ vs. control glomerulonephritis; $^{**}P < 0.0001$ vs. ancrod treatment alone.

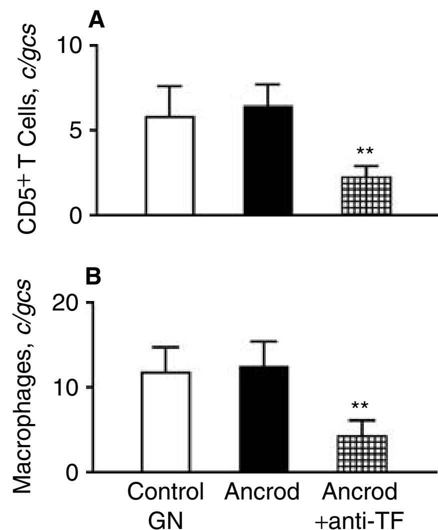

Fig. 4. Glomerular T cells (*A*) and macrophages (*B*) in control treated rabbits with glomerulonephritis (GN), rabbits with glomerulonephritis given ancrod alone and rabbits given ancrod and anti-tissue factor (TF) antibodies. A significant glomerular influx of T cells, (CD5-positive), and macrophages (RAM 11-positive) occurred in experimental crescentic glomerulonephritis. This was unaffected by defibrinogenation with ancrod but significantly affected by the administration of anti-tissue factor antibodies to ancrod-treated rabbits. $^{**}P < 0.001$ vs. control-treated glomerulonephritis or ancrod treatment alone.

much of the glomerular MHC class II expression localized to areas staining for macrophages, MHC class II expression was seen in cells that did not stain positive for RAM 11, suggesting these cells were intrinsic glomerular cells.

DISCUSSION

This study demonstrates that tissue factor has a proinflammatory in vivo effect that is independent of fibrin formation, thus linking the coagulation and inflammatory pathways at the origin of in vivo coagulation. Not only was renal injury prevented as manifest by the preservation of glomerular filtration (measured by serum creatinine), but proteinuria, MHC class II expression as well as macrophage and T-cell infiltration were all decreased by tissue factor inhibition in the absence of pathologic glomerular fibrin deposition. The major effector cell to control the nexus between the coagulation pathway and inflammation is likely to be the macrophage given its marked-up regulation of cell surface–anchored tissue factor during inflammation [10, 21]. In this study the decrease in inflammatory renal injury mediated by blockade of the tissue factor factor VIIa interaction is shown here

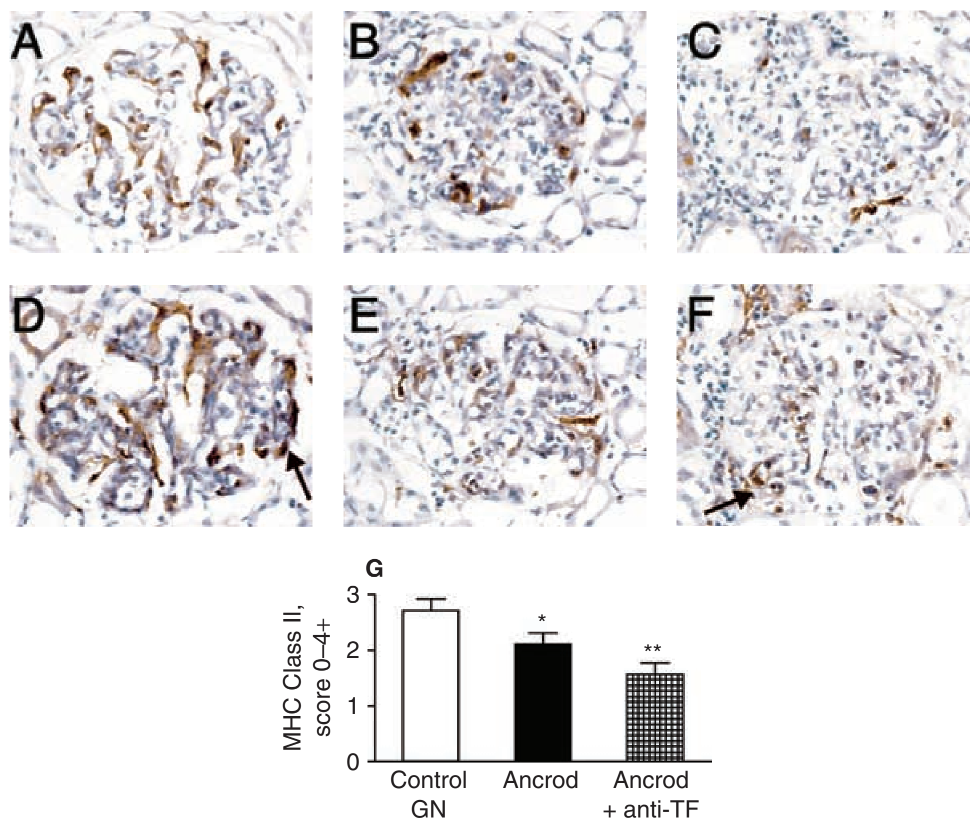

Fig. 5. Glomerular macrophage and major histocompatibility complex (MHC) class II expression in rabbits with glomerulonephritis (GN). Glomerular macrophage accumulation (brown reaction product) was significant both control-treated rabbits (*A*) and rabbits given ancrod alone (*B*), but significantly reduced in rabbits given ancrod and anti-tissue factor (TF) antibodies (*C*). Serial sections showing the same glomeruli showed MHC class II expression (brown reaction product) was prominent in unmodified experimental crescentic glomerulonephritis (*D*). This was reduced by defibrinogenation with ancrod (*E*) and further attenuated by the additional treatment with anti-tissue factor globulin (*F*). While much of the MHC class II expression localized to areas of glomeruli with macrophage accumulation, some segmental areas of glomeruli (D and F, arrows) showed MHC class II expression independent of macrophages. Semiquantitative analysis of MHC class II in glomeruli (*G*) showed a modest reduction with ancrod treatment and a more substantial reduction with ancrod and anti-tissue factor antibodies (serial sections, immunoperoxidase with hematoxylin counterstain, original magnification ×200). $^{*}P < 0.005$ vs. control glomerulonephritis; $^{**}P < 0.005$ vs. both control glomerulonephritis and ancrod treatment alone.

to correlate with a decrease in glomerular macrophage infiltration and is independent of fibrin deposition.

Crescentic glomerulonephritis results from Th1 predominant nephritogenic immune response to antigens in glomeruli [31]. Presentation of nephritogenic antigenic peptides by MHC class II expressing intrinsic glomerular cells is required for the CD4 glomerular influx that recruits and activates the major Th1 effector cells, macrophages [32]. Fibrin deposition plays a relatively minor role in regulation of MHC class II by nephritic glomerular cells as expression was reduced in defibrinogenated animals. However, glomerular leukocyte recruitment was unaffected by defibrinogenation. Tissue factor inhibition was associated with significant reduction in MHC class II expression compared with defibrinogenation and also a significant reduction in T-cell recruitment was observed in rabbits treated with inhibitory anti-tissue factor antibody as well as ancrod. This in turn was associated with a major reduction in macrophage influx. Thus tissue factor and fibrin independently amplify MHC expression. While defibrinogenation with ancrod alone reduced glomerular MHC class II expression, this was relatively minor compared with the greater reduction of MHC class II expression in rabbits treated with both ancrod and anti-tissue factor antibodies. This quantitative difference is likely at least in part, to explain why ancrod alone (unlike both ancrod and anti-tissue factor antibodies together) did not diminish glomerular leukocyte recruitment.

Rabbits were given ancrod prior to the initiation of disease with anti-GBM serum. Treatment with ancrod was shown to significantly inhibit glomerular fibrin deposition with near complete systemic defibrinogenation in both groups of rabbits receiving ancrod as measured by abrogation of glomerular fibrin deposition, serum fibrinogen, the clot quality test of Reid, Chan, and Thean [25] and the observation that the aPTT was unmeasurable in these rabbits. Clinically, this dose of ancrod was

not associated with bleeding during the experimental period nor was there evidence of unexpected hemorrhage at autopsy which is often found at higher doses of ancrod.

Proteinuria was significantly increased in animals treated with ancrod alone. This suggests that heavy fibrin deposition in damaged glomeruli significantly diminished glomerular filtration. Thus abolition of fibrin deposition enhances glomerular clearance rates, its known beneficial effects on renal function (glomerular filtration) confirmed by the significant fall in serum creatinine in ancrod-treated compared with control-treated rabbits with glomerulonephritis. This is consistent with other reports of improved glomerular filtration rates with ancrod treatment of experimental crescentic glomerulonephritis [6, 8]. Interestingly, the augmentation of proteinuria seen in animals treated with ancrod treatment alone is abolished by the additional treatment with anti-tissue factor in these fully defibrinogenated animals. Furthermore, this value is significantly less than that of animals with either control-treated disease or ancrod treatment. This strongly suggests that in crescentic glomerulonephritis, tissue factor in addition to its role in initiating pathologic coagulation is augmenting inflammation by facilitating the expression of MHC class II and directing injurious delayed-type hypersensitvity responses to glomeruli. Ancrod administration has previously been shown to ameliorate disease in this model of glomerulonephritis when used from the initiation of disease [6–9] and as treatment early in the disease [8]. Despite ancrod treatment, decline in renal function still occurs and is associated with the development of massive proteinuria and infiltration of macrophages is unaffected [4]. These studies have suggested that amelioration of the coagulation-dependent impairment in glomerular filtration rate with unmodified inflammatory damage (manifested histologically as mesangial proliferation and cellular infiltration) allows delivery of more protein to immune-injured glomeruli resulting in increased proteinuria. In our studies, ancrod-treated animals had increased proteinuria with no decrease in the number of glomerular T cells and macrophages, further supporting this hypothesis suggesting that defibrinogenation was protective of renal function through its anticoagulant role. The current observations are supported by studies in murine anti-GBM glomerulonephritis using fibrinogen-deficient mice [33]. In these studies, proteinuria was enhanced in fibrinogen-deficient mice with glomerulonephritis in the presence of reduced glomerular fibrin deposition and lower serum creatinine values compared with genetically normal mice with glomerulonephritis [33]. However, in the skin, the deposition of fibrin is important in stable wound healing [34]. Despite the different structure and functions of the dermis and the glomerulus, it is possible that in some circumstances fibrin deposition in glomeruli may play a similar role in providing an initial matrix for subsequent repair [35]. The absence of fibrin in glomeruli coupled with persisting inflammation in rabbits treated with ancrod alone may have contributed to the increased proteinuria in these animals, compared with fibrin intact rabbits.

In the current studies, there was an additional component of protection from deterioration in renal function seen with the addition of tissue factor inhibition suggesting a fibrin independent, delayed-type hypersensitivity–mediated, contribution to the loss of function in this condition. These dual injurious functions of tissue factor were initially suggested by studies of in vivo blockage of the tissue factor/factor VII interaction with anti-tissue factor antibody in coagulation intact rabbits given anti-GBM antibody. This treatment resulted in decreased proteinuria and MHC class II expression compared to control-treated disease [14]. In addition, infusion of TFPI (forming an inactive quaternary complex of tissue factor/factors VIIa and Xa) [12] resulted in not only decreased coagulation and fibrin formation but also in less inflammation in both lipopolysaccharide-induced septic shock models [36] and experimental glomerulonephritis [15]. Tissue factor has been shown to be significantly up-regulated on endothelial cells and macrophages in human and experimental glomerulonephritis. In vivo injection of anti-tissue factor antibodies reduced peritoneal macrophage activation (assessed by reduced reactive oxygen species production and MHC class II expression) [13]. In vitro, human monocyte derived macrophages had factor VIIa augmented triggered reactive oxygen species production and intracellular calcium flux suggesting tissue factor–mediated macrophage activation and intracellular signaling [13].

Precedence exists for the dual proinflammatory and procoagulant roles of coagulation proteases binding to their cell surface receptors. Both the thrombin receptor protease-activated receptor-1 (PAR-1) [37] and endothelial protease receptor-1 (EPR-1) [38, 39], the cell-surface receptor for factor Xa, are constitutively expressed on T cells, macrophages, and endothelial cells. In vitro binding of the protease to its receptor can cause calcium signals and proliferation. In vivo experiments have demonstrated that thrombin receptor–deficient mice, with normal coagulation, are protected from the development of crescentic glomerulonephritis [19] and blocking the EPR-1 factor Xa interaction prevented murine graft versus host disease [40].

CONCLUSION

These studies establish a dual procoagulant and independent proinflammatory function induced by tissue factor factor VIIa binding in an in vivo model of crescentic

glomerulonephritis clearly related to crescentic glomerulonephritis in humans.

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