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A new model of renal microvascular endothelial injury

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A new model of renal microvascular endothelial injury. Although the importance of injury with consequent activation of endothelium is well-recognized in diseases affecting the glomerular endothelial cell (GEN), research on GEN injury *in vivo* has been hampered by the lack of adequate animal models. Here we report the establishment and characterization of a new GEN injury model in rats. This model was induced by selective renal artery perfusion with anti-GEN IgG and resulted in the severe acute renal failure with marked platelet deposition and development of a thrombotic microangiopathy involving glomeruli. Peritubular capillary endothelial cells were also damaged that was associated with severe tubular necrosis. Although the glomerular changes were severe, half of the glomeruli recovered by day 10, while interstitial changes remained throughout our observation time course. Proliferation of GEN was observed during the recovery phase. An increased expression of endothelial nitric oxide synthase in GEN was also observed, and may be an adaptive mechanism to counteract the thrombosis and ischemia. This model should be useful to investigate the pathophysiology of renal microvascular diseases and the mechanisms of GEN injury, activation and recovery *in vivo*.

Vascular endothelium fuctions as a permeability barrier, contributes to the regulation of vasomotor tone, mediates the fluidity of the blood via a number of mechanisms, and is integral to the development of inflammatory foci, both through hemodynamic vasodilator effects and by recruitment of leukocytes. The endothelium may not be merely a target for injury but also is actively involved in the localization and propagation of inflammation. Furthermore, under certain conditions the endothelium may promote a procoagulant state which is likely to be of major importance in the pathogenesis of vascular injury in many disease states. Glomerular endothelium, as well as other endothelium, plays a critical role in the pathophysiology of various disorders such as glomerulonephritis, vasculitis, allograft rejection, ischemia-reperfusion injury, and thrombotic angiopathies. Injury and activation of glomerular endothelial cells (GEN) has been extensively studied *in vitro* [1–8]. However, research on the GEN injury *in vivo* has been hampered by the lack of adequate animal models.

Here we report establishment and characterization of a new rat model of GEN injury.

Key words: endothelium, platelet, hemolytic uremic syndrome, thrombotic microangiopathy, tubulointerstitial disease.

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METHODS

Induction of the GEN injury model

The GEN injury model was induced by selective perfusion of the right kidney through the superior mesenteric artery with the purified IgG of anti-GEN antibody. The technique of selective renal artery perfusion was desribed previously [9]. Kidneys were perfused with 0.2 ml of saline, followed by 80 mg/kg body wt of anti-GEN IgG or control goat IgG in phosphate-buffered saline (PBS), pH 7.2. When a dose response was performed, various doses of IgG was used instead of 80 mg/kDa body wt. Ischemia time was less than six minutes. In separate experiments, as referred to in the **Results** section, various doses of anti-GEN IgG were perfused and the unperfused left kidney was removed prior to closing the abdominal incision.

Immunization of goat with cultured rat GEN

Culture of rat GEN and its characterization has been described elsewhere [10].

A goat was immunized with 1×10^7 rat GEN in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO, USA) and 0.9% saline subcutaneously at multiple sites. The goat was boosted every month with 1×10^7 cells emulsified with incomplete Freund's adjuvant and 0.9% saline. After the second boost the goat was plasmapheresed 10 days following imunization and IgG was purified from the goat plasma as described below.

Purification of goat IgG

Goat IgG for renal artery perfusion was purified utilizing a caprylic acid precipitation method as described before [11, 12]. Briefly, after adjusting the pH of the plasma to 5.5 with HCl, 5.5 ml/100 ml plasma of caprylic acid solution (Sigma) was added dropwise with vigorous stirring. Then the solution was stirred for one hour at room temperature and centrifuged at 14 K rpm for 30 minutes at room temperature. The supernatant was collected and dialyzed against PBS. The dialyzed solution was centrifuged at 14 K rpm for 30 minutes at 4°C. This supernatant was sterilized by filtration and stored at 4°C.

Experimental design

Fifty-two male Wistar rats weighing 200 to 300 grams were used. All animal studies were performed in an accredited animal care facility in accord with the NIH guidelines for the Care and Use of Laboratory Animals.

Renal biopsies were obtained 10 minutes, four hours, one day, three days, and 10 days after the perfusion. At each time point at least four anti-GEnC perfused animals and two control animals were biopsied. Urine was collected from day 0 to day 1, day 1 to day 2, and day 9 to day 10 for urine protein measurements. Serum was obtained at day 1 and day 10 for serum BUN determination. Platelet count and hematocrit was examined prior to and 24 hours after the induction of the model.

Renal histology

Methyl Carnoy's fixed tissue was processed, and 4-μm sections were stained with the periodic acid-Schiff reagent and counterstained with hematoxylin. The numbers of neutrophils, proliferating cells, and macrophages present per glomerular cross-section were counted in a blinded fashion. Thirty randomly selected glomeruli were examined per rat, and the average number of positive cells per glomerulus was calculated. Glomerular cross-sections containing only a minor portion of the glomerular tuft (< 20 discrete capillary segments per cross-section) were not utilized.

Tissue for immunofluorescence was embedded in O. C. T. (Lab-Tek Products; Miles Laboratories, Naperville, IL, USA) and snap-frozen in isopentane.

Antibodies

Glomerular cell proliferation was assessed by staining with 19A2 (Coulter Corp., Hialiah, FL, USA), a mouse monoclonal IgM antibody to the proliferative cell nuclear antigen. Glomerular macrophages were stained with the monoclonal antibody ED-1 (Bioproducts for Science, Indianapolis, IN, USA) at the final concentration of 0.4 μg/ml. GEN were stained with RECA-1, a monoclonal IgG_1 antibody specific for endothelial cells [13]. Glomerular platelet infiltration was assessed by staining with PL-1, a murine monoclonal antibody to rat platelets (a gift from W.W. Baker, University of Groningen, Groningen, the Netherlands) [14] at the final concentration of 5 μg/ml. To stain α-smooth muscle actin, αsm-1 (Boehringer-Mannheim, Germany), a well characterized monoclonal antibody [15], was utilized at the final concentration of 50 ng/ml. Collagen type IV was stained with goat anti-collagen type IV antibody (Southern Biotech, Birmingham, AL, USA) at the final concentration of 2.5 μg/ml. Laminin was stained with rabbit anti-rat laminin antibody (Chemicon, Temecula, CA, USA) at the final concentration of 1.5 μg/ml. Mesangial cells were stained with OX-7 anti-Thy1.1 monoclonal IgG_1 antibody (Serotec Ltd., Oxford, UK) at the final concentration of 0.4 μg/ml. Vascular endothelial growth factor (VEGF) was stained with affinity purified rabbit anti-VEGF polyclonal antibody (Santa Cruz Biotech, Inc., Santa Cruz, CA, USA) [16] at the final concentration of 1 μg/ml. Endothelial nitric oxide synthease (eNOS, NOS III) was stained with mouse anti-eNOS monoclonal IgG_1 antibody (Transduction Lab., Lexington, KY, USA) [17] at the final concentration of 0.08 μg/ml.

To detect proliferation of endothelial cells, double immunostaining was performed as described previously [18]. Briefly, tissue was incubated with RECA-1 (a monoclonal IgG_1) and with anti-PCNA (a monoclonal IgM) followed by biotinylated rabbit anti-mouse IgG_1 (Zymed, San Francisco, CA, USA), avidin D-peroxidase, and diaminobenzidine reaction without nickel chloride, and then peroxidase-conjugated rat anti-mouse IgM (Zymed) antibody, followed by diaminobenzidine containing nickel chloride. Controls included either deletion of the primary antibody or substitution of irrelevant monoclonal antibodies of the same isotype. To detect proliferation of mesangial cells or macrophages, OX-7 or ED-1 was used instead of RECA-1.

Fibrinogen was detected by staining with FITC-conjugated goat anti-fibrinogen IgG (Cappel, Durham, NC, USA) at the final concentration of 25 μg/ml. Rat C3 was detected with FITC-conjugated goat anti-rat C3 (Cappel).

Electron microscopy

Tissue for electron microscopy (EM) was fixed in half-strength Karnovsky's solution (1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.0), postfixed in osmium tetroxide, dehydrated in graded ethanols, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 410 (Philips Export BV, Eindhoven, The Netherlands) electron microscope.

Serum BUN and urinary protein measurement

BUN was determined colorimetrically utilizing a commercial kit for the measurement of urea nitrogen (Sigma Diagnostics, St. Louis, MO, USA).

Urine protein excretion was measured using the sulfosalicylic acid method [19].

Western blotting analysis

Western blotting was performed using the crude extracts of cultured GEN and 10 μg/ml of anti-GEN IgG. The bound antibody was detected with alkaline phosphatase streptavidin (Vector Lab., Burlingame, CA, USA) following incubation 0.75 μg/ml of biotinylated rabbit anti-goat IgG (Zymed). BCIP/NBT tablets (Sigma) were used as a substrate.

Statistical analysis

Data are reported as mean $\pm$ SD. Data are analyzed by Student's t-test with adujstments using the Bonferroni/Dunn method.

RESULTS

Acute renal failure was induced by anti-GEN perfusion

One day after induction of disease, serum BUN levels of anti-GEN perfused rats were significantly higher (25.3 $\pm$ 2.2 mg/dl, $N = 5$) than controls (18.7 $\pm$ 1.9 mg/dl, $N = 4$; $P < 0.005$). BUN levels of anti-GEN perfused rats were still elevated at day 10 (28.0 $\pm$ 4.4, $N = 4$). These rats also demonstrated a slight increase in urinary protein excretion at day 0 to 1 (38.6 $\pm$ 6.6 mg/day, $N = 5$) compared with control animals (21.0 $\pm$ 3.5 mg/day, $N = 4$; $P < 0.005$). Proteinuria in the diseased rats decreased to 21.9 $\pm$ 5.6 mg/day at day1 to 2 ($P < 0.005$). At day 10 the animals perfused with anti-GEN IgG excreted 3.9 $\pm$ 1.0 mg/day of protein ($N = 4$), while the amounts of protein in urine of control rats were 6.5 $\pm$ 1.0 ($N = 2$).

Although the increase in BUN levels described above was statistically significant, the magnitude was small. We speculated that this could be explained by compensation in the left kidney. To confirm this hypothesis, we also perfused uninephrectomized rats with anti-GEN or control IgG. BUN of the uninephrectomized rats perfused with anti-GEN at day 1 was 174 $\pm$ 24 mg/dl ($N = 2$), compared to 31.3 $\pm$ 2.8 in controls ($N = 2$). Furthermore, the increase in BUN in uninephrectomized rats was dependent on the

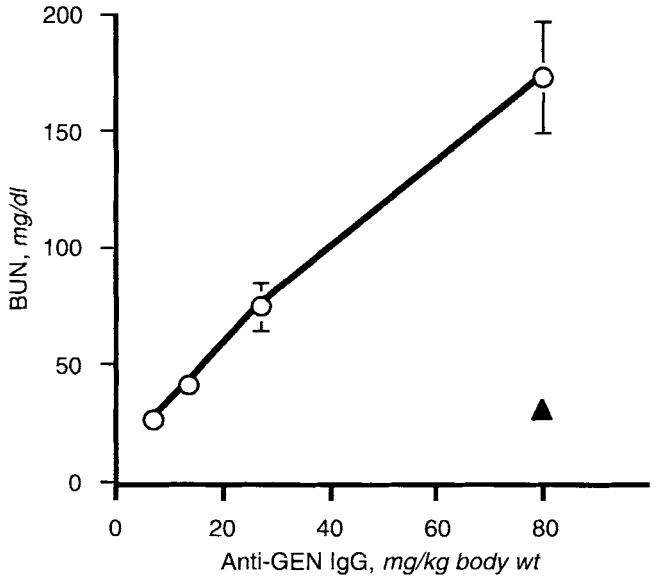

Fig. 1. Increases of serum BUN levels were dependent on the amounts of anti-GEN IgG in uninephrectomized rats. Rats perfused with 80 mg/kg body wt of anti-GEN IgG demonstrated 173.7 ± 23.9 mg/dl of BUN, while BUN levels of the rats perfused with 26.7, 13.3, and 6.7 mg/kg body wt of anti-GEN were 75.5 ± 10.1, 41.9 ± 5.7, 27.0 ± 0.8 mg/dl, respectively (○). Rats perfused with 80 mg/kg body wt of control goat IgG showed 31.3 ± 2.8 mg/dl of BUN (▲). $N = 2$ for each dose.

amounts of anti-GEN IgG administered (Fig. 1). We also measured proteinuria in the uninephrectomized rats perfused with anti-GEN or control IgG at the same dose used for non-nephrectomized rats. Proteinuria in the uninephrecomized rats perfused with 80 mg/kg body wt of anti-GEN did not differ from that of control rats (12.1 ± 1.8 mg/day, and 10.5 ± 0.5, respectively).

Thrombocytopenia and anemia were induced by anti-GEN perfusion

As thrombocytopenia and non-immune hemolytic anemia are common denominators of thrombotic microangiopathy which is also associated with renal microvascular injury, we examined if these features occurred after the perfusion of anti-GEN IgG. Platelet counts and hematocrits prior to the perfusion were not significantly different between the rats perfused with anti-GEN IgG ($N = 4$) and the animals perfused with control goat IgG ($N = 4$; Table 1). One day after perfusion, platelet counts of anti-GEN perfused rats decreased to 71.3 ± 18.1% of the original value, while platelet numbers of control rats remained at 96.8+7.9% ($P < 0.05$). Hematocrits of the anti-GEN perfused rats also decreased to 77.8 ± 5.0%, while those of the control animals were 91.9 ± 1.7% one day after the perfusion ($P < 0.005$). The decrease in hematocrit could be explained by hemolysis. Although our anti-GEN IgG showed a weak cross reactivity with red blood cells (data not shown), we think that hemolysis is mainly due to intravascular mechanical fragmentation of red blood cells, which was suggested by the presence of schistocytes and helmet cells on the peripheral blood films at day 1 (Fig. 2).

Histology of the kidney

Histology of the kidney was examined by PAS staining. Ten minutes after the perfusion, platelet aggregation was observed in the capillary loops of the glomerulus. At day 1 capillary walls were indistinct, and tubular damage was also apparent. Some tubules were necrotic, and white blood cell casts were occasionally observed within the tubular lumens (Fig. 3A). Varying degrees of tubular necrosis was observed, which was often severe and patchy. Arterioles frequently demonstrated denuded or swollen endothelium, and the vasa rectae bundles in the outer medulla were uniformly injured with loss of endothelium. Marked infiltration of polymorphonuclear leukocytes was present in the interstitium (Fig. 3B), while no significant infiltration of these cells was observed in the glomerulus. At day 10, about one half of the glomeruli looked grossly normal, while the other half showed some mesangial expansion. The vasa rectae and swollen arterioles had recovered normal architecture. Larger vessles (interlobular and arcuate) showed an increase in cellularity in the adventitium. Marked atrophy or dilation of the tubules and interstitial fibrosis was also observed (Fig. 3C). None of these features were present in control rats perfused with non-immune IgG.

Silver staining demonstrated that some of the glomeruli of anti-GEN perfused rats were already sclerotic at day 10 (Fig. 3D).

Normal (control) goat IgG perfused rats had normal histology.

Electron microscopic analysis

Within 10 minutes of perfusion with the anti-GEN IgG, glomeruli demonstrated widespread aggregation of platelets within capillary lumina. Many platelets could be seen adherent to the capillary endothelium (Fig. 4). Circulating mononuclear leukocytes were also present in capillary lumina. Capillary endothelium generally maintained a normal appearance, and endothelial fenestrations remained preserved. No evidence of accumulation of fibrin tactoids, immune complex formation, or injury to other glomerular cell types was present. Changes in the tubules were inapparent. Peritubular capillaries also demonstrated widespread accumulation of platelets similar to that encountered in glomerular capillaries.

At four hours, there was little change in the injury to glomerular and peritubular capillaries. Accumulations of platelets were widely persistent. Glomerular endothelium focally demonstrated some degree of swelling and even focal denudation, but generally remained preserved. Mesangial structure and epithelial cell foot processes remained preserved. Immune complex formation and accumulation of fibrin tactoids remained undetectable. The single striking change from the 10 minute biopsies was widespread necrosis of tubular epithelial cells, with extensive sloughing of cells and cell cytoplasm into the tubular lumina.

At twenty-four hours, the accumulations of platelets persisted in the glomerular capillary loops, but in some loops there were now identifiable fibrin thrombi present as well. Red blood cell fragments could be found within the platelet-fibrin mesh in some capillary lumina. Glomerular endothelium was frequently swollen and endothelial denudation from basement membranes more widespread than was present at four hours. In some glomeruli, there was clear evidence of mesangiolysis, with accumulations of electron lucent material mixed with cellular debris present in mesangial regions and extending into the subendothelial space of adjacent capillary walls. Peritubular capillary endothelium showed

Table 1. Platelet counts and hematocrits during the study period

	Prior to disease	Day 1	Decrease ratio
Platelet count of disease rats	896,000 ± 62,600 plts/μl	640,000 ± 17,900 plts/μl	71.3 ± 18.1%
Platelet count of control rats	820,000 ± 42,100 plts/μl	792,000 ± 38,100 plts/μl	96.8 ± 7.9%
Hematocrit of disease rats	50.3 ± 2.4%	39.0 ± 1.4%	77.8 ± 5.0%
Hematocrit of control rats	49.5 ± 1.3%	45.5 ± 1.7%	91.9 ± 1.7%

Abbreviation plts is platelets.

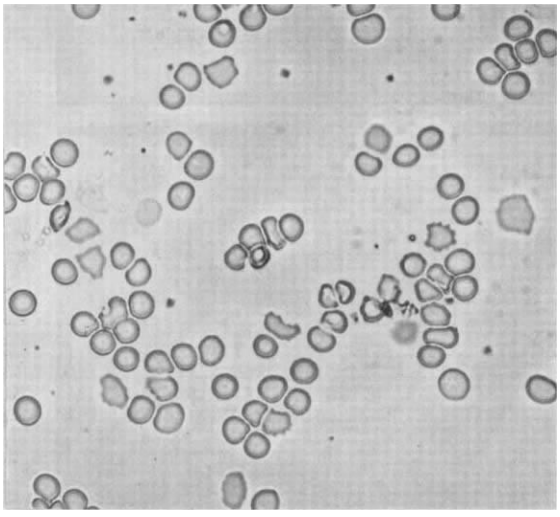

Fig. 2. Signs of erythrocyte fragmentation. Intravascular fragmentation of red blood cells was suggested by the presence of schistocytes and helmet cells on the peripheral blood film at day 1 in anti-GEN antibody perfused rats (magnification ×400).

prominent swelling and focal degenerative changes. Cortical tubules demonstrated a range of appearances from morphologically normal to overt necrosis. At this time point, majority of infiltrating leukocytes were in peritubular capillaries.

At day 3, all features of injury identified at day 1 were still present, but the extent of glomerular capillary thromboses, and the accumulations of subendothelial electron lucent material, were more florid. Mesangiolysis persisted. Extensive tubular necrosis remained evident. About one half of leukocytes were out of the capillaries, while the other half were still in capillary lumina.

By day 10, most of the mesangiolytic injury had resolved. The accumulations of subendothelial electron lucent material were no longer present, and the capillary loops were generally lined by fenestrated, morphologically normal endothelium. In some capillary loops, occasional platelets and monocytes were still present. Some mesangial regions remained modestly expanded due to increased number of cells and some accumulated extracellular material. Peritubular capillaries resumed a normal morphologic appearance. Cortical tubules were largely reconstituted with intact brush borders and complex basolateral interdigitations of cell borders.

Anti-GEN IgG was localized along the glomerular capillaries

The glomeruli of the animals perfused with anti-GEN IgG demonstrated bright linear staining in a capillary pattern with anti-goat IgG antibody at 10 minutes (Fig. 5A). The staining intensity did not change at four hours and at day 1. The staining with anti-goat IgG decreased in intensity at day 3. The glomeruli were still positive at day 10, although the fluorescence intensity was faint.

The peritubular capillaries and arterioles in the kidney were also positive at 10 minutes, four hours, and day 1. The non-glomerular renal microvascular staining decreased substantially after four hours. Large vessels (interlobular) and venous endothelium showed minimal staining at all time points.

In order to find out exactly what antigens this polyclonal anti-GEN antibody is directed toward, Western blotting analysis utilizing crude extracts of cultured GEN was performed. The blot showed multiple bands (data not shown), and we could not deduce what was the most prominent antigen for this antibody by this analysis.

GEN injury

GEN morphology was assessed by staining with the endothelial cell specific monoclonal antibody, RECA-1. The staining pattern remained unchanged 10 minutes after the perfusion. At four hours some glomeruli showed a decrease in RECA-1 staining, although most glomeruli demonstrated a normal staining pattern. At day 1 most glomeruli showed decreased RECA-1 staining to some extent (Fig. 6 A, B), consistent with GEN injury. At day 3 approximately one half of the glomeruli recovered an intact RECA-1 staining, while the rest still showed a decrease of the staining. At day 10 all glomeruli demonstrated an intact RECA-1 staining pattern.

Platelet and neutrophil infiltration

Marked platelet aggregation within glomerular capillaries was observed at 10 minutes after the perfusion (Fig. 6 C, D). Intense staining with anti-platelet antibody persisted until day 1. At day 3 many glomeruli were free of anti-platelet staining, although there were some glomeruli that still had prominent platelet aggregation within them. At day 10 no anti-platelet staining was observed in the glomeruli. Platelet infiltration was also observed in the peritubular capillaries. Such infiltration was localized only in the cortical area. Platelet aggregation in muscular arteries was much less than that observed in microvessels.

In contrast to the marked platelet infiltration, there was no significant neutrophil infiltration within glomeruli at any of the time points investigated, although marked interstitial neutrophil infiltration was observed at day 1 and day 3 in association with leukocyte casts (Fig. 3).

Deposition of fibrin

Fibrin was already deposited in glomeruli 10 minutes after the perfusion (Fig. 5B). Four hours later deposition of fibrin had increased, and at day 1 and day 3 most glomeruli showed

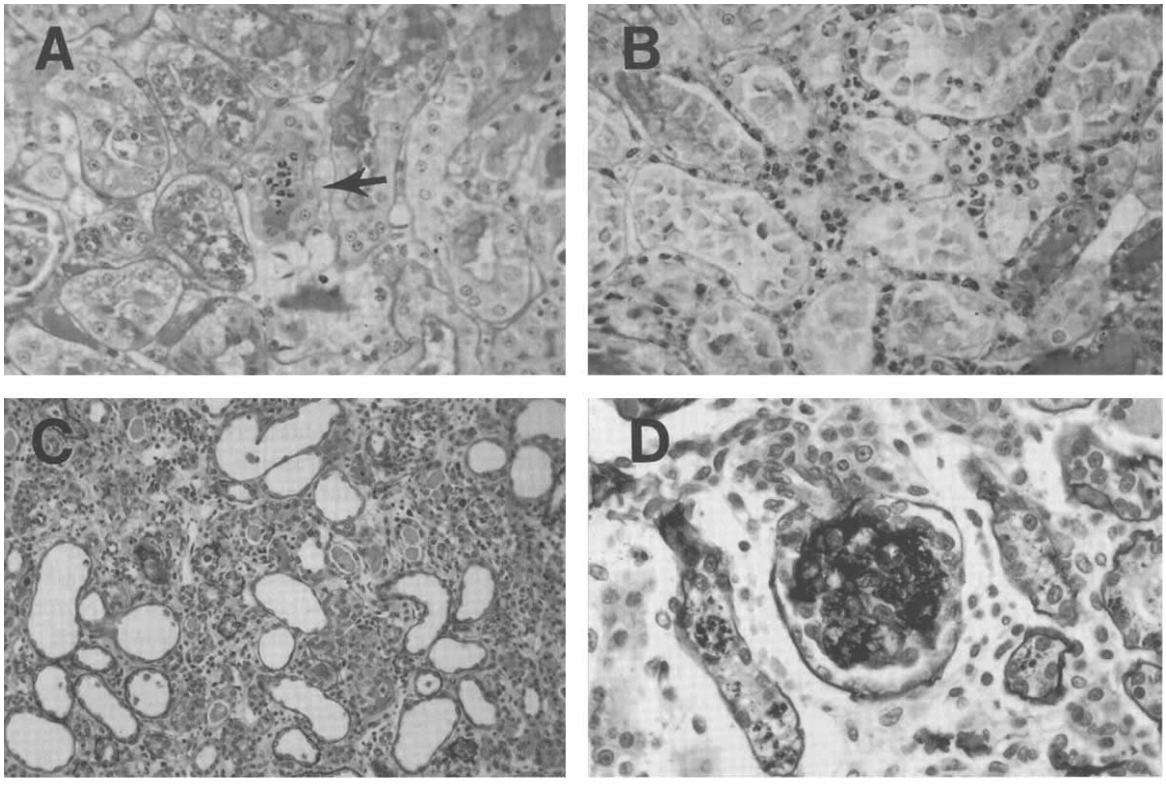

Fig. 3. Histology of the kidney. (*A*) White blood cell casts were observed within the tubular lumens at day 1 (PAS staining). (*B*) Marked infiltration of polymorphonuclear leukocytes was observed in the interstitium at day 1 (PAS staining). (*C*) Severe tubulointerstital damage, including tubular dilatation and atropy, was observed at day 10 (Silver staining). (*D*) Some of the glomeruli demonstrated focal sclerosis at day 10 (Silver staining; magnification ×400).

prominent fibrin deposition. At day 10 no fibrinogen deposition was observed in glomeruli.

Deposition of complement components in the glomerulus

Deposition of C3 was observed along the capillary walls at 10 minutes (Fig. 5C). Intensity of C3 staining decreased four hours after the perfusion, and no C3 deposition was observed 24 hours after the perfusion. No staining of C3 was observed in control glomeruli.

Macrophage infiltration

A significant infiltration of ED-1 positive macrophages in glomeruli was observed only at day 3 (4.0 ± 1.0 ED-1 positive cells/glomeruli in the anti-GEN perfused rats, $N = 4$, vs. 1.3 ± 0.1 in the control rats, $N = 2$, $P < 0.001$; Fig. 7).

Four hours after the induction of disease a few infiltrating macrophages were observed in the interstitium. At day 1 interstitial macrophage infiltration dramatically increased, and persisted at day 3. At day 10 the interstitial macrophage infiltration was still prominent.

Mesangial area

To examine if the mesangial cells were also involved in this model, we investigated the distribution pattern of cells positive for OX-7 (Thy1) staining, a marker of mesangial cells, and expression of α-smooth muscle actin, a marker of mesangial cell activation.

At day 1 the mesangial areas appeared normal, and there were no glomeruli that expressed α-smooth muscle actin. However, at day 3 mild mesangial expansion was observed with OX-7 staining (Fig. 8A), compared to control (Fig. 8B). Some glomeruli showed α-smooth muscle actin expression at day 3 (Fig. 8C), although the expression level was much lower than that observed in the anti-Thy1 mesangioproliferative glomerulonephritis model of rats (data not shown). Distribution of mesangial cells returned to normal and no α-smooth muscle actin expression was observed at day 10. It should be noted that there were many α-smooth muscle actin positive cells in the interstitium at day 3 and day 10 (Fig. 8D).

Glomerular cell proliferation was increased at day 3

Glomerular cell proliferation was assessed by PCNA staining. At day 1 no significant increase in cell proliferation was observed. A significant increase of PCNA positive cells in glomeruli was observed only at day 3 (5.5 ± 2.0 PCNA positive cells/glomeruli in the anti-GEnC perfused rats, $N = 4$, vs. 0.2 ± 0.1 in the control rats, $N = 2$, $P < 0.001$). To determine which glomerular cells were proliferating, we performed double staining with PCNA and RECA-1, OX-7, or ED-1. No proliferation of macrophages was detected in the glomerulus. Most of the PCNA positive cells were endothelial cells (Fig. 9), but a small portion of these cells were mesangial cells (OX-7 positive). We also observed proliferation of peritubular capillary endothelial cells and tubular cells (Fig. 9). No significant glomerular cell proliferation was demonstrated at day 10.

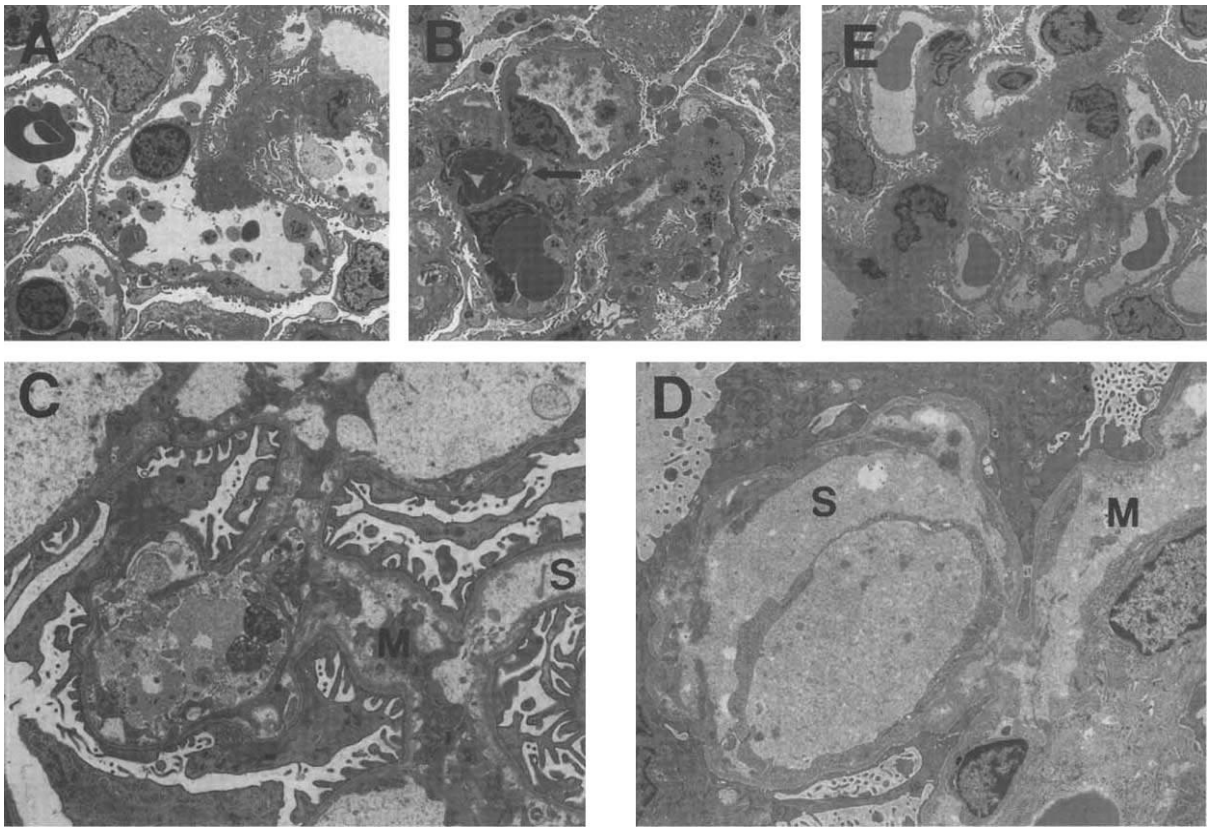

Fig. 4. Electron microscopic analysis. (*A*) 10 minutes after perfusion with the anti-GEN IgG, many platelets could be seen adherent to the capillary endothelium. Capillary endothelium generally maintained a normal appearance, and endothelial fenestrations remained preserved. (*B*) One day after perfusion, persistent accumulations of platelets were observed in the glomerular capillary loops. Intracapillary accumulations of finely fibrillar fibrin tactoids were now apparent (arrow). Red blood cells and red blood cell fragments were also found within the platelet-fibrin mesh in some capillary lumina. (*C*) One day after perfusion, glomerular endothelium was swollen and endothelial denudation from basement membranes was widespread. Mesangiolysis (M) was also observed in some glomeruli, with accumulations of electron lucent material mixed with cellular debris present in mesangial regions and extending into the subendothelial space (S) of adjacent capillary walls. (*D*) At day 3, accumulation of electron lucent material in the disrupted mesangial regions (M) was still apparent. There was now widespread extension of this material into the subendothelial space (S) of adjacent capillary walls, similar to the glomerular injury characteristic of human thrombotic microangiopathy. (*E*) At day 10, most glomeruli recovered normal morphologic features with normal endothelial appearances. A few residual platelets could be detected in some capillary lumina.

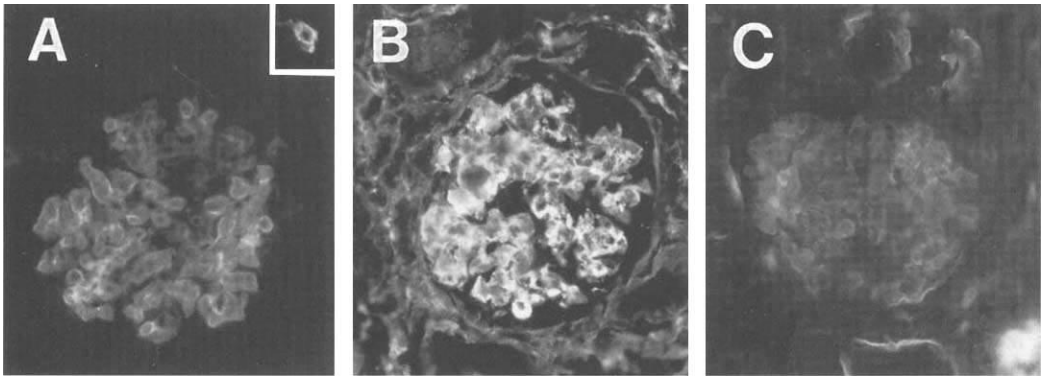

Fig. 5. Glomerular deposition of anti-GEN IgG, fibrinogen, and C3 by immunofluorescence. (*A*) 10 minutes after the perfusion, anti-GEN IgG was clearly demonstrated on the capillary walls in the glomerulus in a linear pattern. Deposition of anti-GEN IgG was also demonstrated in the peritubular capillaries (*inset*). (*B*) Marked deposition of fibrinogen was observed 10 minutes after the perfusion. (*C*) C3 deposition was observed on the capillary walls 10 minutes after the perfusion (magnification ×400).

VEGF expression in the glomerulus was increased at day 1

As the glomerulus was recovering GEN staining (RECA-1) at day 3 and the peak of cell proliferation (PCNA) was observed at the same period, we speculated that some growth factors might be inducing recovery by stimulating GEN proliferation. Therefore, we examined the expression of one of the well-known endothelial

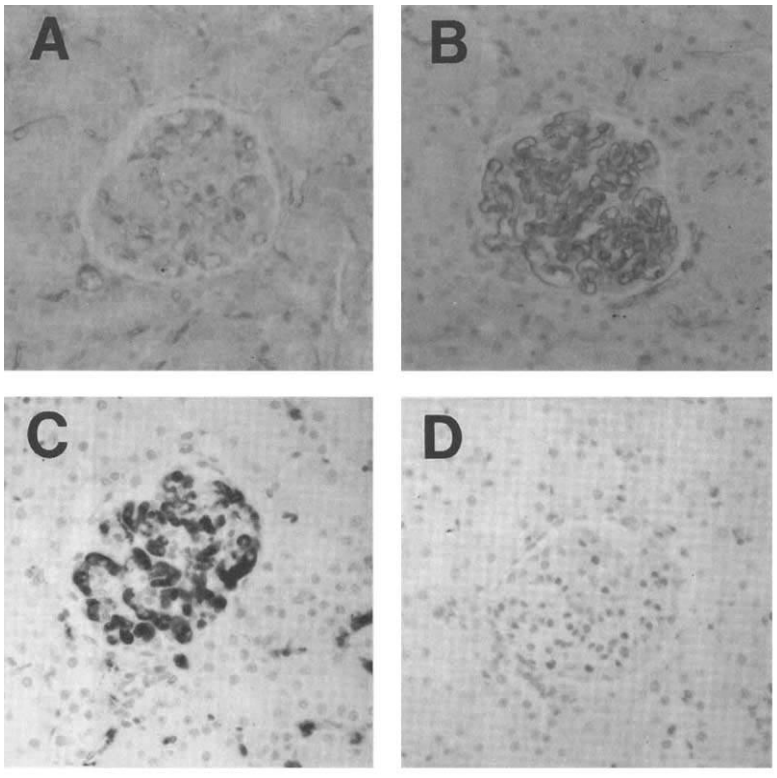

Fig. 6. Staining of GEN and platelets. (*A*) At day 1 most glomeruli showed a decrease in GEN staining as determined by RECA-1 staining. (*B*) Control rats showed intact RECA-1 staining along the glomerular capillary walls at day 1. (*C*) Marked aggregation of platelets was observed 10 minutes after the perfusion by PL-1 staining. (*D*) No aggregation of platelets was observed in control rats (magnification ×400).

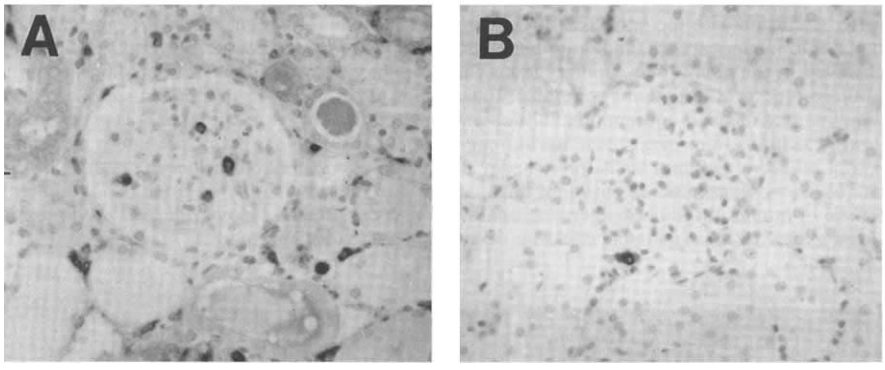

Fig. 7. Macrophage infiltration. (*A*) Significant macrophage infiltration was observed at day 3 by ED-1 staining. It should be noted that macrophage infiltration was also prominent in the interstitium. (*B*) No significant increase of macrophage infiltration was observed in control rats (magnification ×400).

cell growth factors, VEGF. We observed a marked increase of VEGF production in the glomerulus at day 1, which appeared to be primarily localized to the glomerular epithelial cells (Fig. 10 A, B).

eNOS was up-regulated by GEN

Nitric oxide (NO) is a well-known mediator of various biological phenomenon, including as a vasodilator and as an inhibitor of platelet aggregation. Thus, we performed staining with anti-eNOS antibody. We observed significant up-regulation of eNOS at four hours and day 1 (Fig. 10 C, D). The staining pattern suggested that eNOS was being expressed by the GEN.

Extracellular matrix protein

Marked deposition of type IV collagen in the glomerulus was observed at day 1 and day 3 (Fig. 11). At day 10 type IV collagen levels in one half of the glomeruli returned to normal. In contrast, type IV collagen level in the interstitium dramatically increased at day 10.

Laminin deposition in the glomerulus was also increased at day 1 and day 3 (Fig. 11). At day 10 laminin expression of one half of the glomeruli returned to normal. In contrast, laminin deposition in the interstitium increased at day 3 and persisted until day 10.

A summary of these immunohistochemical analyses described above are shown in Table 2.

Other organs

We also examined liver, lung, spleen, and heart of anti-GEnC perfused and control rats. These organs from the animals perfused with anti-GEnC IgG showed positive staining of microvascular endothelium with anti-goat IgG compared with control rats, although the staining was very faint. No tissue damage was detected by H/E staining of these organs.

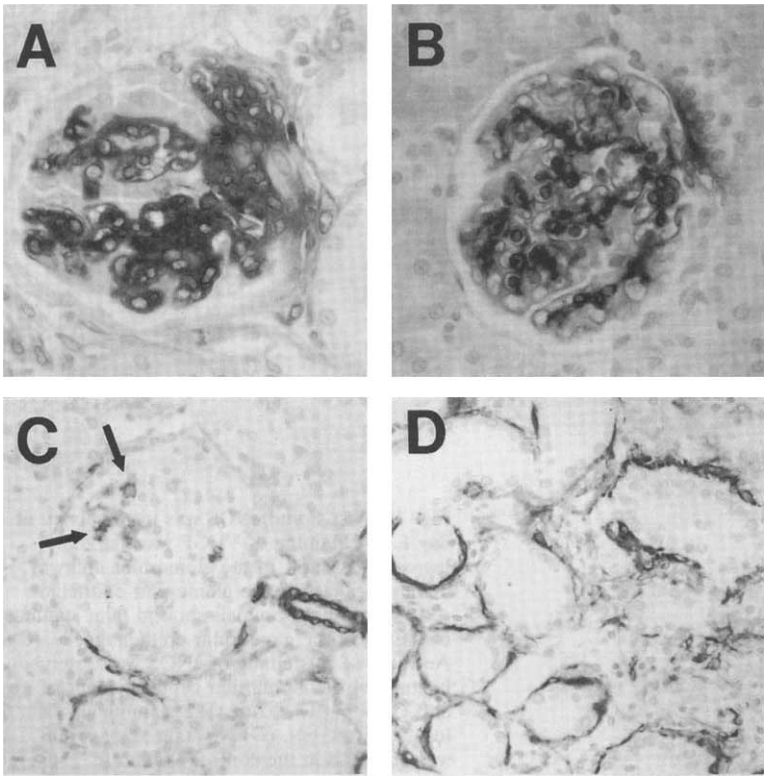

Fig. 8. Mesangial changes. (*A*) OX-7 (Thy1) staining showed mild mesangial expansion in some glomeruli at day 3. (*B*) Control rats showed a typical mesangial pattern of OX-7 staining. (*C*) In a small number of glomeruli, a slight expression of α-smooth muscle actin was observed at day 3. (*D*) Many α-smooth muscle actin expressing cells were observed in the interstitium at day 10 (magnification ×400).

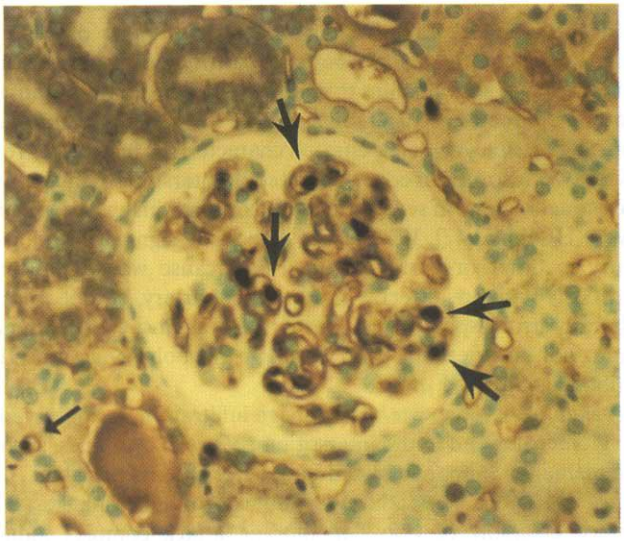

Fig. 9. Double staining with RECA-1 and PCNA. Indirect immunoperoxidase double-immunostaining for PCNA (represented by the black nuclear stain) and RECA-1, a marker for endothelial cells (represented by the brown cytoplasmic stain), was performed. A representative glomerulus at day 3 of anti-GEN perfused rats showed that most PCNA-positive cells were GEN (examples are indicated by the broad arrows). Proliferation of peritubular capillary endothelial cells was also observed (represented by the thin arrow) (magnification ×400).

DISCUSSION

We report a new model of GEN injury. A major problem in GEN injury research has been a lack of animal models. Among the most important glomerular diseases in which GEN are a principal target of injury is renal thrombotic microangiopathy (TMA) such as hemolytic uremic syndrome (HUS). HUS shows acute GEN injury that results in renal TMA with platelet consumption and thrombocytopenia, fibrin deposition, non-immune hemolytic anemia with fragmented erythrocytes, and renal failure [20–23]. Most cases of epidemic HUS are associated with enteric infection with verotoxin-containing *E. coli* O157, in which it is believed that the verotoxin mediates the disease [20, 24, 25]. Although a verotoxin-induced model of HUS might be ideal, the Gb3 receptors required for binding of the toxin are not present on rabbit GEN (as opposed to human GEN), and therefore injection of verotoxin has not induced renal disease in rabbits [26]. To our knowledge, nobody has succeeded in making HUS-like lesions in rats by injection of verotoxin, either. Furthermore, HUS also occurs in the setting of other infections (such as Shigella, or human immunodeficiency virus), drugs (mitomycin C, cyclosporine), severe hypertension, and pregnancy, or can be idiopathic. Thus, the development of a model of GEN injury would be very useful to elucidate the mechanisms of GEN injury, activation, and recovery.

Several GEN injury models have been previously reported. Matsuo et al reported glomerulonephritis induced by anti-angiotensin converting enzyme antibody administration in rabbits [27]. However, due to the paucity of expression of the antigen within the glomerulus, the disease was mild and transient. Matsuda induced glomerular disease by immunizing guinea pigs with the membrane products from cultured brain endothelial cells [28]. However, the disease was also mild and was accompanied by neurological symptoms.

The two best models available so far utilized implanted antigen-antibody reactions on the glomerular capillary walls. The first one

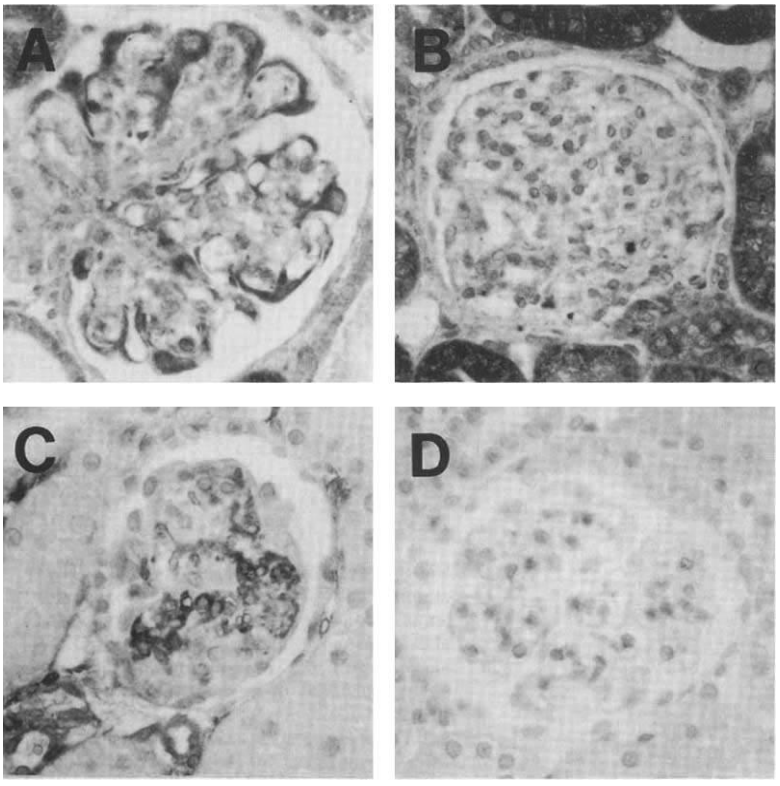

Fig. 10. VEGF and eNOS was up-regulated at day 1. (*A*) Staining of VEGF was markedly increased at day 1 in the glomerulus and was mainly localized in the glomerular epithelial cells. (*B*) Control animals showed faint staining of VEGF in the glomerular epithelial cells. (*C*) An increase in staining of eNOS was observed in some of the glomerular capillary loops of anti-GEN perfused rats. The staining was localized to GEN. (*D*) No staining of eNOS was observed in the control glomeruli (Magnification ×630).

is the concanavalin A (con A) model, which was originally reported by Golbus and Wilson [29]. This is induced by perfusion of a renal artery of rats with con A, a protein extract from the jack bean *Concanavalia ensiformis*, followed by antibody to con A. Con A, which binds to the glomerular capillary wall [30], serves as a planted antigen in the initiation of GEN injury. This model is characterized by platelet and leukocyte infiltration [9, 31–34]. The second model, which was developed by Matsuo's group, utilizes another lectin, *Lens culinaris hemoagglutinin*, and anti-lectin antibody [35]. This model also results in GEN injury with platelet and leukocyte infiltration. However, these models do not lead to arteriolopathy and patchy cortical necrosis as observed in TMA.

Although our model is immunologically induced, which is not believed to be the case in the classical post-diarrheal HUS due to *E. coli*, our model demonstrated many features of renal microangiopathy. It should also be noted that HUS can also be secondary to a variety of other disorders or be idiopathic, as described above. Furthermore, although HUS is a typical disorder which shows renal microangiopathy, various other disorders including SLE, progressive systemic sclerosis, a primary antiphospholipid syndrome, and malignant hypertension demonstrate similar pathological features of renal microangiopathy [4, 36–44]. Renal microangiopathy is known to be a high risk factor of progressing to end-stage renal disease in SLE [45]. Although the mechanism of GEN injury in our model may be different from the classic HUS, we believe that the GEN response to injury and many other sequential events are similar, and that therefore this model will be very useful to elucidate the pathologenic cascades which occur following GEN injury in TMA and other renal microangiopathies.

A main feature of our model was acute renal failure, which was clearly demonstrated by the histology and high BUN values in uninephrectomized rats. The extremely high value of BUN in uninephrectomized rats can be partly attributed to intravascular hemolysis that accompanies this model. The lack of proteinuria in the uninephrectomized rats may reflect the severe renal failure. Histological analysis demonstrated characteristic features of renal TMA. We also observed marked tubulointerstitial damage with occasional regions of severe tubular necrosis, which is also seen in severe HUS [46, 47]. We did not detect any signs of histological injury in other organs. This may be because we administered anti-GEN antibody directly into the renal artery, and because of relatively strong specificity of our antibody against GEN, although we do not know exactly what antigens this polyclonal anti-GEN antibody is directed toward.

A significant monocyte/macrophage infiltration was observed in the glomerulus and in the interstitium. Various cytokines, such as complement components and monocyte chemoattractant protein-1 (MCP-1), can induce chemotaxis of macrophages. Cell surface assembly of C5b-9 membrane attack complex induces MCP-1 secretion from endothelial cells [48]. It was also demonstrated that glomerular endothelial cells can produce MCP-1 *in vitro* [49]. Considering the time course of macrophage accumulation, it is possible that some cytokines such as MCP-1 released by GEN, rather than complement components, induced macrophage infiltration in this model. Fibrin deposition and associated production of fibrin degradation products can also facilitate attraction and activation of macrophages.

Although we observed mononuclear cells in capillary lumina at 10 minutes, the number of ED-1 positive cells in glomeruli did not show a statistically significant increase at that time point. However, the number of ED-1 positive cells at 10 minutes (2.2 ± 1.0 per glomerulus) was twice that of control rats (1.1 ± 0.7), and we

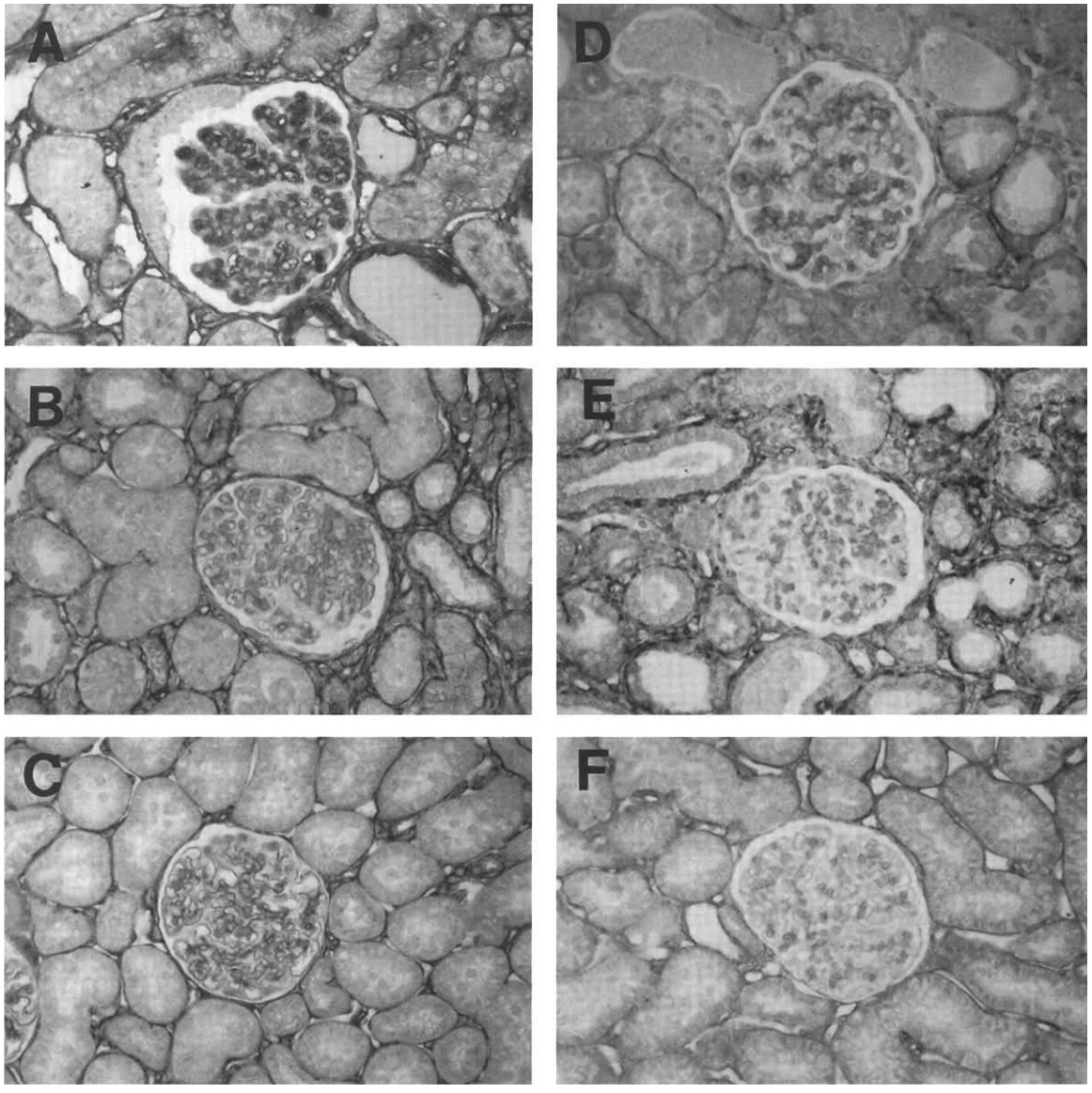

Fig. 11. Extracellular matrix protein. Accumulation of collagen type IV and laminin was observed in the glomeruli of anti-GEN perfused rats at day 1 (collagen type IV, *A*; laminin, *D*) and day 3. Deposition of collagen type IV and laminin was demonstrated in the interstitium of anti-GEN perfused rats at day 3 and day 10 (collagen type IV, *B*; laminin, *E*). Control rats showed no increase of collagen (*C*) or laminin (*F*) (magnification ×400).

believe that the limited number of animals in each group may have limited the ability to detect a statistical significance.

Interestingly, in contrast to previously reported GEN injury models [9, 31–33, 50, 51], we did not observe neutrophil infiltration in the glomerulus.

GEN damage was suggested by decrease of staining with anti-endothelial cell antibody RECA-1 at day 1 and day 3. However, RECA-1 staining and glomerular morphology recovered at day 10. This suggests that loss of GEN was followed by a recovery with GEN proliferation. Consistent with this, we observed significant cell proliferation at day 3. Double staining suggested most of these proliferating cells were GEN.

Several growth factors, including VEGF, have been shown to stimulate GEN proliferation *in vitro* [52, 53]. VEGF, also called vascular permeability factor (VPF), is a mitogen for endothelial cells [54–56]. It was demonstrated that ^{125}I-labeled VEGF binds to the capillaries of glomeruli [57], and that glomerular endothelial cells have receptors for VEGF [58]. Here we observed upregulation of VEGF in glomerular epithelial cells at day 1. VEGF was detected in podocytes, which was consistent with previous findings [18, 59–62]. This localization makes interpretation of the role of up-regulated VEGF difficult given the flux of the ultrafiltrate that will hamper the binding of podocytic VEGF to the receptors on GEN on the other side of the GBM.

Interestingly, expression of α-smooth muscle actin was observed in the glomerulus at day 3. The *de novo* expression of α-smooth muscle actin is considered to be a marker of activation of mesangial cells [63]. The mechanism of induction of α-smooth muscle actin in this GEN injury model is unclear, but it may be a result of "cross-talk" between mesangial cells and GEN. The

Table 2. Immunochemical analyses

	10 Minutes	4 Hours	Day 1	Day 3	Day 10
Goat IgG	++	++	++	+	+
Platelet	++	++	++	+ ~ −	−
Fibrin	+	++	++	++	−
RECA-1	N	N ~ dec	dec	N ~ dec	N
PCNA			N (0.4 ± 0.1)	+ (4.5 ± 2.1)	N (0.1 ± 0.1)
Macrophage					
glom	N (2.2 ± 1.0)	N (1.1 ± 0.4)	N (1.2 ± 0.3)	+ (3.5 ± 0.9)	N (1.0 ± 0.1)
int	N	N ~ +	++	++	++
α-SM actin					
glom			−	− ~ +	−
int			N	+	+
VEGF		N	++	N	N
eNOS		+	+	N	N
Collagen IV					
glom			++	++	N ~ +
int			+	+	++
Laminin					
glom			++	++	N ~ +
int			+	+	++

Abbreviations and symbols are: N, normal range; −, not detected; +, up-regulated; ++ markedly up-regulated; dec, decreased; glom, glomerular; int, interstitial.

injured GEN may have induced phenotypic changes of the mesangial cells by releasing cytokines [64]. The *in vitro* modulations of mesangial cells by cytokines derived from endothelial cells have already been reported [65, 66]. Matsuo's group also reported proliferation of mesangial cells in their GEN injury model that utilized an exogenous implanted antigen and they speculated that the mechanism could be generation of growth factors by the infiltrated cells [35].

α-Smooth muscle actin expression was also observed in the interstitium. Induction of smooth muscle characteristics in the interstitial cells of the kidney was thought to be due to the "myofibroblast-like" phenotypic change of interstitial fibroblasts [67, 68]. In addition, it was demonstrated that this alteration parallelled the interstitial macrophage number, which suggested that infiltrating macrophages elaborated some cytokines such as TGF-β and exerted a paracrine effect on resident interstitial fibroblasts to acquire a new phenotype [68]. It should be noted that α-smooth muscle actin expression in the interstitium parallelled the macrophage infiltration in our model as well. Similar mechanisms may be working in our model.

We also examined the regulation of eNOS in this model, because endothelium-derived relaxing factor/nitric oxide (NO) is one of the major thromboregulatory systems in endothelial cells [69, 70]. NO not only causes smooth muscle relaxation and consequent vasodilation, but also inhibits platelet adhesion and aggregation [71–76]. NO has been shown to play an important role to prevent glomerular thrombosis in the pregnant state or with endotoxin challenge [77–79]. Furthermore, clinical improvement by administration of NO donor was reported in one case of HELLP syndrome (hemolysis, raised liver enzymes, and low platelets), one of a range of conditions characterized by thrombotic microangiopathy that includes HUS [80]. eNOS was previously shown to be expressed in GEN [81, 82]. In the ATS models of rats, a subtle increase of eNOS mRNA was detected by ribonuclease protection assay [83]. Although we do not have data on its enzymatic activity, our immunohistochemical data in this study suggested up-regulation of eNOS in the glomerulus. We speculate that eNOS was up-regulated in order to compensate for platelet aggregation and ischemia. Up-regulation of constitutive NOS in the kidney was also observed in norepinephrine-induced acute renal failure rats [84]. Furthermore, elevation of plasma concentrations of the NO metabolites, which was speculated to be a result of eNOS induction, was reported in patients with HUS [85].

In conclusion, we established a new rat model of renal microvascular endothelial cells. This model showed severe acute renal failure with marked platelet deposition in the glomerulus in association with thrombocytopenia and anemia with fragmented erythrocytes. Peritubular capillary endothelial cells were also damaged, accompanied by severe tubular necrosis. Although the glomerular change was very severe, one half of the glomeruli recovered at day 10, while interstitial change leading to fibrosis remained throughout our observation time course. Proliferation of GEN was observed during the recovery stage. Up-regulation of eNOS in the glomerulus, which we think is a compensatory mechanism for the ischemia, was observed. We believe this model will serve an important tool to investigate the pathophysiology of renal microvascular diseases.

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