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Tumor Necrosis Factor Induces Glomerular Damage in the Rabbit

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Tumor necrosis factor (TNF) is a polypeptide hormone produced by activated macrophages detectable in the circulation of experimental animals given endotoxin. Recent evidence strongly suggests that many of the deleterious effects of endotoxin in experimental animals are mediated by TNF. Because endotoxemia in experimental animals and humans is associated with glomerular damage the present investigation was designed to establish whether TNF directly induces glomerular functional and structural changes. Twenty-three rabbits were given human recombinant TNF at the doses of 0.08, 0.8, and 8.0 μg/kg/h as a continuous 5-hour intravenous infusion. Animals were killed at the end of the infusion. All rabbits given 0.8 and 8.0 μg/kg/h TNF developed anemia (Ht value decrease at 5 hours: 0.8 μg/kg/h, 15%; 8.0 μg/kg/h, 16%); leukopenia (leukocyte count decrease at 5 hours: 0.8 μg/kg/h, 47%; 8.0 μg/kg/h, 59%); thrombocytopenia (platelet count decrease at 5 hours: 0.8 μg/kg/h, 45%; 8.0 μg/kg/h, 57%). Rabbits given 8.0 μg/kg/h also had renal failure (serum creatinine from 1.02 ± 0.15 to 1.64 ± 0.34 mg/dl). By light microscopy only occasional polymorphonuclear leukocytes in the glomerular capillaries were detectable in rabbits infused with 0.08 μg/kg/h TNF, whereas with 0.8 μg/kg/h TNF the presence of inflammatory cells in the glomerular capillaries was the prominent finding. With 8.0 μg/kg/h TNF beside leukocyte accumulation, fibrin was detected in the glomerular capillary lumens of two of eight animals. Electron microscopy found dose-dependent glomerular endothelial cell damage in animals given TNF with fibrinlike material in the capillary lumens. Glomerular changes induced by TNF were remarkably similar to those previously found in animals given endotoxin. Thus, TNF is likely to be the mediator of endotoxin-induced glomerular damage and can be regarded as a new mediator of macrophage-dependent damage in glomerulonephritis. (Am J Pathol 1989, 134: 419–430)

Injection of endotoxin in experimental animals is accompanied by hypotension, disseminated intravascular coagulation, and widespread tissue damage.[1–3] Evidence from bone marrow transplant experiments indicates that endotoxin is not responsible for inducing such changes per se but does promote the generation of endogenous mediators that appear to be responsible for shock and death in experimental animals.[4] Recently a macrophage-derived polypeptidic hormone, tumor necrosis factor (TNF),[5,6] has been purified,[7–9] cloned, and sequenced.[10–12] TNF is the likely candidate for mediating the clinical manifestations and tissue damage that follow endotoxin injection. Thus, in rabbits injected with endotoxin TNF has been detected in the circulation at nanomolar concentrations.[8,13] Moreover, when injected in rats, TNF induced hypotension, metabolic acidosis, hemoconcentration, and death.[14] Finally, mice passively immunized against TNF are protected from the lethal effect of endotoxin.[15]

The aim of the present investigation was to establish whether TNF can directly induce glomerular damage. Our results document that the infusion of TNF to New Zealand White rabbits is associated with the development of glomerular changes, which include glomerular endothelial damage, polymorphonuclear cell accumulation in the capillary lumens and fibrin formation, reminiscent of those found previously in rabbits given endotoxin.[16] These data provide further support for the concept that the deleterious effects of endotoxin in experimental animals are mediated by TNF. Moreover, they suggest that TNF can now be regarded as a potential new mediator of injury in glomerular diseases.

Materials and Methods

Experimental Design

Male New Zealand White rabbits (Charles River Italia s.p.a., Calco, Italy) weighing 1.8–2.3 kg were used. Hu-

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man recombinant TNF was the gift of Dr. Leo S. Lin (Cetus Corporation, Emeryville, CA). The specific activity of TNF was 10^7 U/mg. TNF was virtually endotoxin free (<66 ng/mg by limulus amebocyte lysate assay). TNF was diluted in pyrogen-free normal saline containing 0.1% bovine serum albumin (BSA, Sigma Chemical Company, St. Louis, MO). Endotoxin contamination of BSA-saline preparation as checked by limulus amebocyte lysate assay was <0.062 ng/ml. Three groups of animals were intravenously infused with TNF at doses of 0.08 (group 1, N = 5), 0.8 (group 2, N = 10), and 8.0 (group 3, N = 8) μg/kg/h for 5 hours. An additional group (group 4, N = 5) was infused with identical volume of saline-BSA (vehicle) for 5 hours and served as a control.

TNF and vehicle were infused into an ear vein of nonanesthetized animals and infusions were maintained constant via a syringe pump at the rate of 1 ml/h. Blood samples were drawn from contralateral ear arteries at 0, 3, and 5 hours for measurement of hematocrit, platelet count, white blood cell count, and serum creatinine. Plasma TNF levels were measured in five of eight rabbits given the highest dose of TNF before and at the end of the infusion. In four rabbits of group 3 renal plasma flow (RPF) was measured by the clearance of p-aminohippuric acid (PAH) during 5-hour saline-BSA (vehicle) infusion, and 7 days later during 5-hour TNF infusion (8.0 μg/kg/h). All animals were killed at the end of the 5-hour infusion. Kidneys were removed and tissue specimens processed for light and electron microscopy. Isolated glomeruli were prepared from kidneys of rabbits infused with the highest doses of TNF (0.8 and 8.0 μg/kg/h) or with vehicle to evaluate the production of TxB2, the stable metabolite of TxA2, and 6-keto-PGF1α, the stable metabolite of PGI2. The morphology of ear veins after infusion with the highest dose of TNF was examined in three rabbits and no ultrastructural changes were found. The effect on renal morphology of longer follow-up studies after the end of TNF infusion was examined in an additional group of ten rabbits (group 5) that was given TNF at the dose of 8.0 μg/kg/h for 5 hours. Rabbits were killed at hours 15 and 24 after infusion ended (five rabbits for each time point) and kidney specimens processed for light and electron microscopy. In the same animals 15- or 24-hour urine samples were collected in metabolic cages for urinary protein excretion measurement.

Endotoxin contamination of TNF preparations was ruled out as a cause of glomerular injury by two sets of experiments. Boiling the infusion mixture eliminated TNF activity, but TNF, unlike endotoxin, is heat-labile, and polymyxin B inhibited endotoxin but not TNF activity. In particular, three rabbits were intravenously infused for 5 hours with 8.0 μg/kg/h TNF boiled for 60 minutes, and three rabbits were infused with the same dose of TNF treated with polymyxin B (1 μg/ml) (Sigma Chemical Company). Additionally, three rabbits were intravenously infused for 5 hours with endotoxin (Bacto Lipopolysaccharide [LPS] *Escherichia coli* 0111:B4, Difco Laboratories, Detroit, MI) at a dose of 0.5 ng/kg/h, which approximates the level of endotoxin measured in the TNF-BSA-saline preparation. Endotoxin was dissolved in 5 ml of pyrogen-free normal saline just before the infusion procedure.

To verify whether TNF could have a direct toxic effect on glomerular cells, isolated glomeruli from kidneys of normal rabbits were incubated in Dulbecco's minimum essential medium (D-MEM, GIBCO, Paisley, UK) in the presence or absence of TNF. TNF was added to glomeruli at the concentrations of 0.005, 0.05, and 0.5 μg/ml and lactate dehydrogenase (LDH) release measured after 5, 15, and 24 hours incubation at 37 C.

To assess whether TNF induces a glomerular damage directly or whether the effect of TNF was mediated by circulating cells we used the system of *in situ* isolated rat kidney. Kidneys were perfused in a recirculating system at a constant pressure of 95–100 mmHg with an artificial cell-free medium containing TNF or vehicle. After a 15–20 minute equilibration period, TNF (8.5 and 85 ng/min) or vehicle was continuously infused into the renal artery via a syringe pump over a 90-minute period. At the end of the experiment specimens from TNF- and vehicle-exposed kidneys were obtained for light and electron microscopy studies.

To verify whether the effects of TNF on glomerular structure was mediated by interleukin-1, rabbits (N = 3) were infused intravenously for 5 hours with human recombinant interleukin-1 (IL-1) β (8.0 μg/kg/h). In an additional three rabbits, IL-1β (8.0 μg/kg/h) was infused together with TNF (8.0 μg/kg/h). IL-1β (kindly provided by Dr. Boraschi, Sclavo, Siena, Italy) had a specific activity of 10^7 U/mg and contained 0.5 pg endotoxin/μg. IL-1β was diluted in pyrogen-free normal saline containing 0.1% BSA. At the end of the infusion rabbits were killed. Kidneys were removed and tissue specimens processed for light and electron microscopy.

Biochemical Analysis

Serum creatinine was measured by the alkaline picrate method.[17] For plasma TNF measurement, blood was collected in 5 ml EDTA tubes in the presence of aprotinin (67.5 μl Trasylol; 20,000 Kallikrein inhibiting units/ml; Bayer Italia s.p.a., Milano, Italy). After centrifugation at 450*g* for 10 minutes, platelet-rich plasma was transferred to Eppendorf tubes and spinned in a microfuge for 1 minute to remove platelets. Platelet-poor plasma was then aspirated and stored frozen at −20 C. TNF was measured by a commercially available ELISA kit (T cell Sciences, Inc., Cambridge, MA). Proteinuria was determined by the

modified Coomassie blue G dye-binding assay for proteins[18] with BSA as standard.

Renal Plasma Flow Measurement

RPF was estimated by measuring the clearance of PAH (Sigma Chemical Company) in unanesthetized rabbits and securely held in a plastic restraining cage, according to Gunther and Rabinowitz[19] with minor modifications. Urine was continuously collected from the bladder by way of a rubber retention catheter in graduated cylinder. The catheter was inserted through the urethra when the animal was under light anesthesia by penthotal sodium (Abbot s.p.a., Aprilia, Rome, Italy) administered through a catheter inserted in one lateral ear vein. After recovery from anesthesia, a saline solution (3 ml) containing 3% PAH was infused in the ear vein through a 21-gauge needle attached to a length of polyethylene tubing, as a priming load, followed by constant infusion of the same solution via a syringe pump (Hospal, Bologna, Italy) at 4.2 ml/hour. A 45-minute equilibration period was allowed after initiation of infusion to attain constant plasma level of PAH. Five clearance periods of 60 minutes each were performed. Blood samples of 0.7–1 ml were collected into a heparinized syringe at the midpoint of urine collection periods from a catheter placed in the central artery of a contralateral ear. The arterial catheter was a 21-gauge needle attached to polyethylene tubing. Plasma was separated by centrifugation. Plasma and urine PAH were determined by a method described previously.[20] At the end of each experiment, the bladder was washed out with a dilute solution of nitrofurazone to control possible bladder infection. All intravenous solutions were sterilized by filtration through a 0.45-μ pore filter.

Isolation of Glomeruli

Kidneys were quickly removed under pentobarbital sodium anesthesia from rabbits and placed in ice-cold KRB, pH 7.4. All subsequent preparative steps were performed at 4 C. Renal capsules were removed and the cortex separated from the medulla and finely minced to a pastelike consistency. The homogenate was gently pressed through a series of 180-μ (80 mesh) and 125-μ (120 mesh) sieves, which excluded most of the tubuli, and then washed with KRB, pH 7.4, over a 105-μ (140 mesh) sieve that retains glomeruli. Glomeruli were washed twice in KRB, pH 7.4, and centrifuged at 150*g* for 5 minutes. The purity of isolated glomeruli was determined microscopically by counting the number of glomerular and nonglomerular particles suspended in a given volume. The purity of the final pellet, consisting of decapsulated glomeruli, was 80–90%.

Incubation of Glomeruli

Glomeruli from rabbits infused with TNF or vehicle were resuspended in 1 ml KRB supplemented with $CaCl_2$ (2.5 mM) and incubated at 37 C for 20 minutes in a shaking bath. At the end of the incubation, the glomeruli were centrifuged at 2000*g* for 10 minutes, and the supernatants were removed, frozen, and stored at −20 C until assayed for TxB2 and 6-keto-PGF1α. Protein content of glomerular preparations was determined according to the method of Lowry et al[21] using BSA (Sigma Chemical Co.) as a standard.

Radioimmunoassay

TxB2 and 6-keto-PGF1α, the stable hydrolysis products of TxA2 and PGI2, respectively, were measured by RIA of samples diluted in 20 mM phosphate buffer. Six thousand disintegrations per minute of tritiated TxB2 (120 Ci/mmol) or 6-keto-PGF1α (150 Ci/mmol) (Amersham International plc, Buckinghamshire, UK), and variable dilutions of specific rabbit antisera sufficient to bind 40% of the homologous tracer (final dilutions, 1:250,000 for TxB2 antiserum, 1:300,000 for 6-keto-PGF1α antiserum) were incubated 16–24 hours at 4 C in a final volume of 1.5 ml. Separation of free from antibody-bound labeled antigen was obtained by rapid addition of 0.1 ml of human PG-free plasma and 0.1 ml of a charcoal suspension (100 mg/ml) (Norit A, Serva, Feinbiochemia, Heidelberg, FRG), which adsorbs 95–98% of free PGs and subsequent centrifugation for 20 minutes at 2000*g* at 4 C. Supernatant solutions containing antibody-bound TxB2 or prostaglandin were decanted directly into 8 ml of Instagel (Packard Instrument Co. Inc., Downers Grove, IL). Radioactivity of samples was counted in a liquid scintillation counter (LS 1800 model, Beckman, Irvine, CA). The smallest concentration that could be measured with 95% confidence was 2 pg/ml for all the antisera. The cross-reactivity of the antisera at 50% displacement was, for anti-TxB2 antiserum, 2,3 dinor-TxB2: 10.5%; 6-keto-PGF1α: 0.006%; PGE2: 0.007%; PGD2: 0.02%; PGF2α: 0.023%; AA: <0.004%; for anti-6-keto-PGF1α antiserum, 2,3 dinor-6-keto-PGF1α: 16.6%; 6,15 diketo PGF1α: 0.22%, 13,14-dihydro-6,15-diketo-PGF1α: 0.075%; PGF2α: 3%; PGD2: 0.09%; 2,3-dinor-TxB2: 0.02%; PGE2: 0.37%; TxB2: <0.006%; leukotriene B4 (LTB4): 0.015%; leukotriene C4 (LTC4): <0.01%; AA: 0.0023%.

Results were expressed as nanograms per milligram of glomerular protein per 20 minutes. Intra-assay and in-

terassay variability of RIA measurements evaluated by assays of several glomerular preparations averaged 3 and 4% respectively. Validation of RIA measurements was obtained by several independent criteria: dilution and recovery studies, comparison among multiple antisera, characterization of the chromatographic pattern of distribution of the extracted TxB2, PG-like immunoreactivity on thin-layer chromatography (TLC), and comparison with gas chromatographic mass spectrometric determinations.

LDH Release

The supernatants from incubations of TNF with isolated glomeruli were measured for LDH release according to a method described previously.[22]

Isolated Perfused Kidney

The perfusion technique used in these experiments was a modification of that described by Roblero et al[23] and Vio et al.[24] Sprague-Dawley rats (Charles River Italia s.p.a., Calco, Italy), 300–400 g body weight, were anesthetized by an intraperitoneal injection of thiopental sodium (50 mg/kg body weight) and placed on a heated surgical table. The right kidney and retroperitoneal structures were exposed through a midline abdominal incision. After the abdominal cavity was exposed, the following steps were performed: 1) The adrenal artery, which arises from the right renal artery, was identified and tied. 2) The vena cava tributaries below the right renal pedicle and above the iliac bifurcation were tied. Additional loose ligatures were placed around the vena cava just above the right renal pedicle and below the right renal vein. 3) After intravenous injection of 0.5 ml of 10% mannitol and 120 IU heparin, the right ureter was isolated from the surrounding connective tissue and cannulated with PE-10 polyethylene tubing (Clay-Adams, Parsippany, NJ). 4) The open tip of a venous cannula (PE-240 polyethylene catheter) with its distal end closed was introduced into the vena cava below the right renal vein and secured in place. 5) The distal end of the superior mesenteric artery was tied and loose ligatures were placed around the superior mesenteric artery and the right renal artery near the aorta. Care was taken to avoid excessive manipulation of the renal pedicle. 6) The renal artery was cannulated with a short, blunted 19-gauge needle, via the superior mesenteric artery to avoid interruption of flow to the kidney. 7) The arterial cannula was then secured in place by the previously placed ligatures, the distal end of the venous cannula was opened, and the ligature around the vena cava above the right renal pedicle was tied. 8) The kidney was washed with 50 ml of perfusion solution until the venous effluent was free of blood, as determined by the absence of the peroxidaselike activity of hemoglobin (Hemastix strip, Miles GmbH, Sparte AMES, Frankfurt, FRG). Then the venous effluent was recirculated.

Venous outflow was collected into a small glass beaker and returned to a reservoir (closed system) through a two-headed Minipulse peristaltic pump (Gilson 2, Villier Le Bel, France). The perfusion apparatus consisted of a plastic reservoir bottle from which the perfusion medium passed to hollow-fiber membrane oxygenator (Bellco, Mirandola, Italy). The perfusate was drawn through the blood compartment of the oxygenator, and a gas mixture (95% O_2 to 5% CO_2) continuously passed through the gas compartment, oxygenating the perfusion solution and holding its pH at 7.35–7.40. From the oxygenator, the medium passed through Tygon tubing to the second head of the peristaltic pump, an in-line 8-μ filter (Sartorius, Gottingen, FRG), and a glass bubble trap. Finally the perfusate was delivered through latex tubing to the renal artery cannula.

The perfusion medium was maintained at 37 C by a constant Haake D1 temperature circulator (Haake, Berlin, FRG). Perfusion pressure was continuously measured with a Statham transducer (Gould, Dusseldorf, FRG) connected by polyethylene tubing to the perfusion line proximally to the arterial cannula and recorded on a Battaglia Rangoni KV 135 recorder (Battaglia Rangoni, Casalecchio di Reno, Bologna, Italy). Perfusion pressure was corrected by the pressure generated by the catheter alone at each flow rate, and variations in perfusion pressure were compensated for by changing the rate of the peristaltic pump. Because the system was maintained at constant pressure (95–100 mmHg), changes in perfusate flow reflected changes in renal vascular resistance.

The perfusate consisted of Krebs-Henseleit buffer (120 mM NaCl, 25 mM $NaHCO_3$, 2.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 1.5 mM $MgSO_4$) containing 3.5 g/100 ml Ficoll 70 (Pharmacia Fine Chemicals, Uppsala, Sweden), 1.0 gm/100 ml bovine serum albumin (Pentex BSA Fraction V, Miles Laboratories, Elkhart, IN), 200 mg/100 ml dextrose, 36 mg/100 ml of urea, 50 mg/100 ml creatinine, and the following L-amino acids in mM: methionine, 0.5; alanine, 2.0; glycine, 2.0; serine, 2.0; arginine, 1.0; isoleucine, 1.0; aspartic acid, 3.0; cysteine, 0.5. This perfusate was filtered through a 0.45-μ membrane filter (Sartorius) before use and, when equilibrated with the gas mixture at 37 C, its pH was approximately 7.4. The total volume of the perfusate was 250 ml.

Light Microscopy

Fragments of renal cortex were fixed in Dubosq-Brazil fluid (80% alcohol, 150 ml; formol, 60 ml; acetic acid, 15

ml; picric acid, 1 g) and embedded in paraffin. Sections of 2 μ were stained with hematoxylin and eosin (H & E), Masson's trichrome, periodic acid-Schiff staining (PAS), and Fraser-Lendrum staining for fibrin. Each biopsy included at least 100 glomeruli. Glomerular polymorphonuclear leukocyte infiltration was expressed as the total number of polymorphonuclear leukocytes per glomerulus counted in 30 glomeruli for each animal and expressed as the mean ± SD. Fibrin deposits, revealed by Fraser-Lendrum staining, were graded from 0 to 4+ (0, no fibrin; 1+, 1–25% of glomeruli with fibrin deposits; 2+, 25–50% of glomeruli with fibrin deposits; 3+, 50–75% of glomeruli with fibrin deposits; 4+, 75–100% of glomeruli with fibrin deposits). All renal biopsies were analyzed by the same pathologist blind to the nature of the experimental groups.

Electron Microscopy

Fragments of kidney were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 4 hours at 4 C. Samples were washed in cacodylate buffer with 5% sucrose and subsequently postfixed in 1% osmium tetroxide for 1 hour. They were dehydrated through ascending grades of alcohol and embedded in Epon. Sections were cut on an LKB V ultramicrotome (LKB Instruments, Milan, Italy). Semi-thin sections were stained with 1% toluidine blue in borax and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined with a Zeiss EM 109 (Carl Zeiss, Oberkochen, FRG). At least 4 glomeruli for each animal were analyzed.

Statistical Analysis

The results are expressed as the mean ± SD. Biochemical data (variables: dose and time) were analyzed by two-way analysis of variance using the Tukey-Cicchetti's test for multiple comparison. Data of glomerular production of TxB2 and 6-keto-PGF1α were analyzed by one-way analysis of variance by using Duncan's multiple range test. Histologic data were analyzed by using the Mann-Whitney U test. P values < 0.05 were considered significant.

Results

Biochemical Parameters

Rabbits given 5-hour continuous infusion of vehicle or human recombinant TNF at the dose of 0.08 μg/kg/h did not develop fever and conjunctival inflammation, whereas these signs were present in rabbits infused with 0.8 and

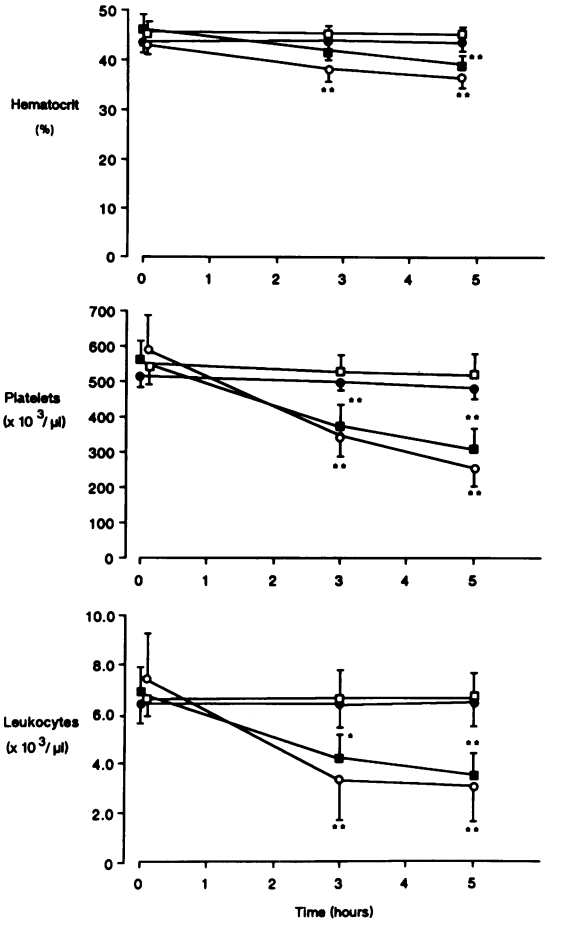

Figure 1. *Hematocrit, platelet, and leukocyte counts of rabbits infused with vehicle (□) or 0.08 (●), 0.8 (■), 8.0 (○) μg/kg/h TNF for 5 hours. Results are expressed as mean ± S.D. *P < 0.05, **P < 0.01 vs. vehicle of the corresponding time.*

8.0 μg/kg/h TNF. Rabbits given vehicle or 0.08 μg/kg/h TNF did not show any drop in hematocrit values (Figure 1). A significant and dose-dependent decrease in hematocrit during the study period was observed in rabbits given 0.8 and 8.0 μg/kg/h TNF compared with controls. Platelet counts dropped suddenly after the TNF infusion at the highest doses and remained lower than control values for all the infusion period (Figure 1). Rabbits given vehicle or 0.08 μg/kg/h TNF showed a normal leukocyte count during all the infusion period, whereas the white blood cell counts significantly decreased in rabbits infused with 0.8 and 8.0 μg/kg/h TNF compared with controls. Serum creatinine concentration remained within normal values for all the study period in rabbits given vehicle or 0.08 and 0.8 μg/kg/h TNF (Figure 2). In contrast, a rise in the serum creatinine occurred in rabbits treated with 8.0 μg/kg/h TNF, which reached a statistical significance at the end of the infusion period (Figure 2). Plasma TNF levels measured in five rabbits before (basal) and at the

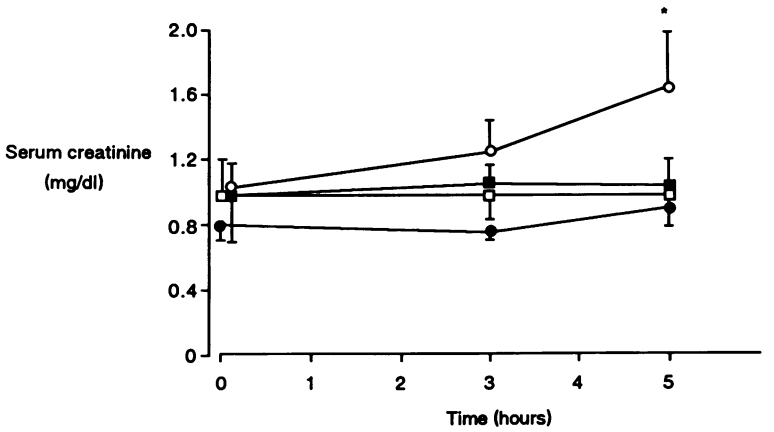

Figure 2. *Serum creatinine of rabbits infused with vehicle (□) or 0.08 (●), 0.8 (■), 8.0 (○) μg/kg/h TNF for 5 hours. Data are expressed as mean ± SD.* *P < 0.05 vs. *vehicle of the corresponding time.*

end of 5-hour infusion with 8.0 μg/kg/h TNF were <0.040 and 283.7 ± 139.11 ng/ml, respectively.

Proteinuria

Urinary protein excretion was measured before and at the end of the experimental period in rabbits treated with 8.0 μg/kg/h TNF and killed 15 and 24 hours after the end of infusion. At both times proteinuria was the same as the respective basal values (at 15 hours: 19.3 ± 5.7 *vs.* 16.3 ± 1.7 mg/15 h; at 24 hours: 19.6 ± 9.1 *vs.* 15.4 ± 4.4 mg/day).

Renal Plasma Flow

Table 1 shows RPF during vehicle or TNF infusions (8.0 μg/kg/h) monitored in the same animals 7 days apart. Vehicle as well as TNF infusions did not result in any changes in RPF throughout the 5-hour experimental period. Furthermore, no significant differences were found in RPF during TNF infusion compared with vehicle at each time interval considered.

Glomerular Production of TxB2 and 6-keto-PGF1α

Glomerular production of TxB2 and 6-keto-PGF1α were evaluated in rabbits infused with the highest doses of TNF (0.8 and 8.0 μg/kg/h) or vehicle and killed after 5-hour infusion. No difference in glomerular TxB2 and 6-keto-PGF1α production in respect to vehicle values was observed after TNF treatment with both doses considered (Table 2).

Glomerular LDH Release

LDH release from isolated glomeruli incubated with TNF was not different from that of controls independent of the concentration of TNF used and the incubation time. LDH release ranged from 0 to 4% of control values.

Light Microscopy

Animals injected with vehicle did not show any significant changes in glomeruli, tubules, and vessels (Figure 3). Rabbits infused with 0.08 μg/kg/h TNF disclosed only occasional polymorphonuclear leukocytes in the glomerular capillaries. In rabbits infused with 0.8 μg/kg/h TNF the main pathologic finding was characterized by the presence of inflammatory cells mainly consisting of polymorphonuclear leukocytes in the glomerular capillaries (7.3 ± 1.9 polymorphonuclear leukocytes/glomerulus, Figure

Table 1. *Effect of 5-Hour Vehicle or TNF Infusion on Renal Plasma Flow (RPF)*

		RPF (ml/min·kg body wt)				
	Time (hours)	1	2	3	4	5
Vehicle	mean	27.20	21.30	23.90	27.10	24.10
	±SD	7.00	7.80	8.20	4.62	1.80
TNF	mean	23.50	26.00	24.00	20.90	20.90
	±SD	6.00	6.30	3.90	3.40	6.50

Table 2. *Glomerular Production of TxB2 and 6-keto-PGF1α in Rabbits Infused for 5 Hours with Vehicle or TNF (0.8 and 8.0 μg/kg/h)*

	TxB2 (ng/mg prot)	6-keto-PGF1α (ng/mg prot)
Vehicle	2.94 ± 1.30	3.77 ± 1.18
TNF (0.8 μg/kg/h)	2.63 ± 1.64	3.03 ± 1.09
TNF (8.0 μg/kg/h)	2.98 ± 1.34	4.99 ± 0.24

Results are expressed as mean ± SD.

4). By Lendrum staining no fibrin deposits were detected in the glomeruli. In rabbits infused with 8.0 μg/kg/h TNF 7.8 ± 0.5 polymorphonuclear leukocytes/glomerulus were detected. In two of eight animals fibrin deposits in a few glomerular capillaries were found by Lendrum staining. Some polymorphonuclear leukocytes also were observed in the peritubular capillaries. Tubular, interstitial, arterial, and arteriolar changes were unremarkable in all rabbits studied. In the additional group of rabbits studied at 15 hours after the end of infusion with 8.0 μg/kg/h TNF the accumulation of inflammatory cells was significantly reduced (3.1 ± 0.4 polymorphonuclear leukocytes/glomerulus, $P < 0.002$) when compared with that observed at 5 hours and no fibrin was detected in the glomerular capillary lumens. Twenty-four hours after the end of TNF infusion (8.0 μg/kg/h) no significant glomerular damage was observed in all animals.

The infusions of boiled TNF and 0.5 ng/kg/h endotoxin did not induce any evident pathologic changes; in contrast when TNF treated with polymyxin B was infused to rabbits the usual pattern of polymorphonuclear leukocyte accumulation in the glomeruli was detected, demonstrating that glomerular changes are not due to a contamination of TNF with endotoxin, but to a specific effect of TNF itself. The infusion of TNF in a free blood system of isolated and perfused kidney failed to induce any significant damage of glomerular structure. In the group of animals treated with IL-1 (8.0 μg/kg/h) a mild accumulation of inflammatory cells in the glomerular capillaries was observed (3.7 ± 0.1 polymorphonuclear leukocytes/glomerulus). These values were significantly lower ($P < 0.01$) than those observed infusing the same amount of TNF (see above). The simultaneous infusion of TNF (8.0 μg/kg/h) and of IL-1 (8.0 μg/kg/h) promoted inflammatory cell accumulation in the glomeruli that was slightly but significantly higher ($P < 0.01$) than in rabbits treated with TNF alone (9.7 ± 0.2 polymorphonuclear leukocytes/glomerulus) but there were no fibrin deposits.

Electron Microscopy

Control animals showed no significant ultrastructural changes in the glomerular capillary loops (Figure 5), tubules, interstitium, and vessels. Rabbits infused with 0.08 μg/kg/h TNF revealed only mild endothelial cell swelling and loss of fenestrae. In rabbits infused with 0.8 μg/kg/h TNF glomerular endothelial cells displayed a focal loss of fenestrae and mild cytoplasmic swelling. Moreover, the glomerular capillary lumens contained several polymorphonuclear leukocytes (Figure 6). Glomerular deposits of fibrinlike material were never detected. In animals infused with 8.0 μg/kg/h TNF changes of glomerular endothelial cells were markedly worse, with the loss of fenestrae more severe and the swelling of cytoplasm more extensive (Figure 7). In one animal the endothelium was de-

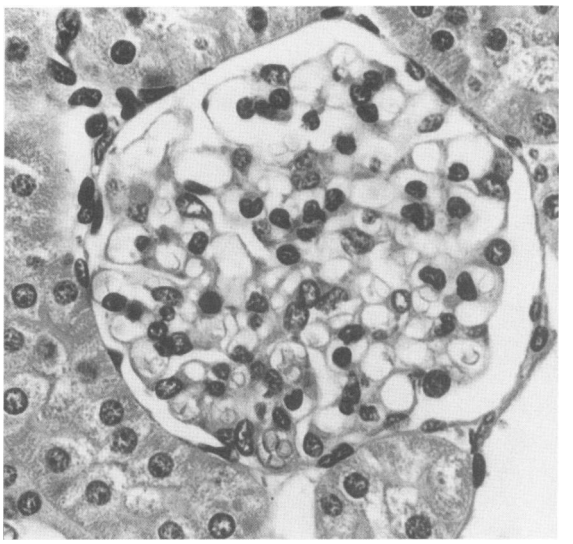

Figure 3. *Rabbit infused with vehicle for 5 hours. The glomerulus does not show any significant pathologic changes (Trichrome ×400).*

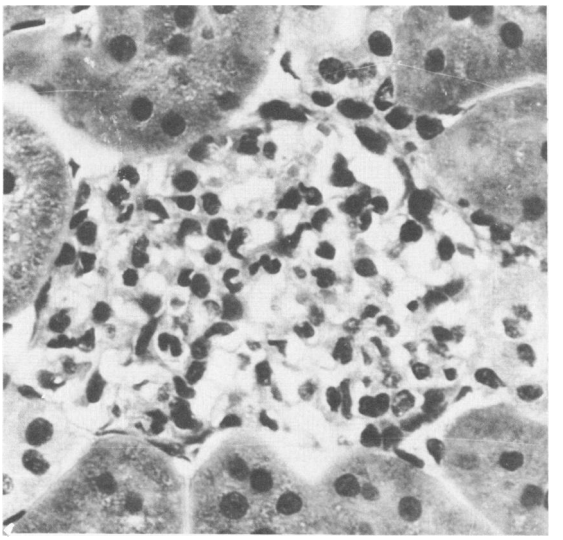

Figure 4. *Rabbit infused with 0.8 μg/kg/h TNF for 5 hours. Note the presence of several polymorphonuclear leukocytes in the glomerular capillary lumens (Trichrome ×400).*

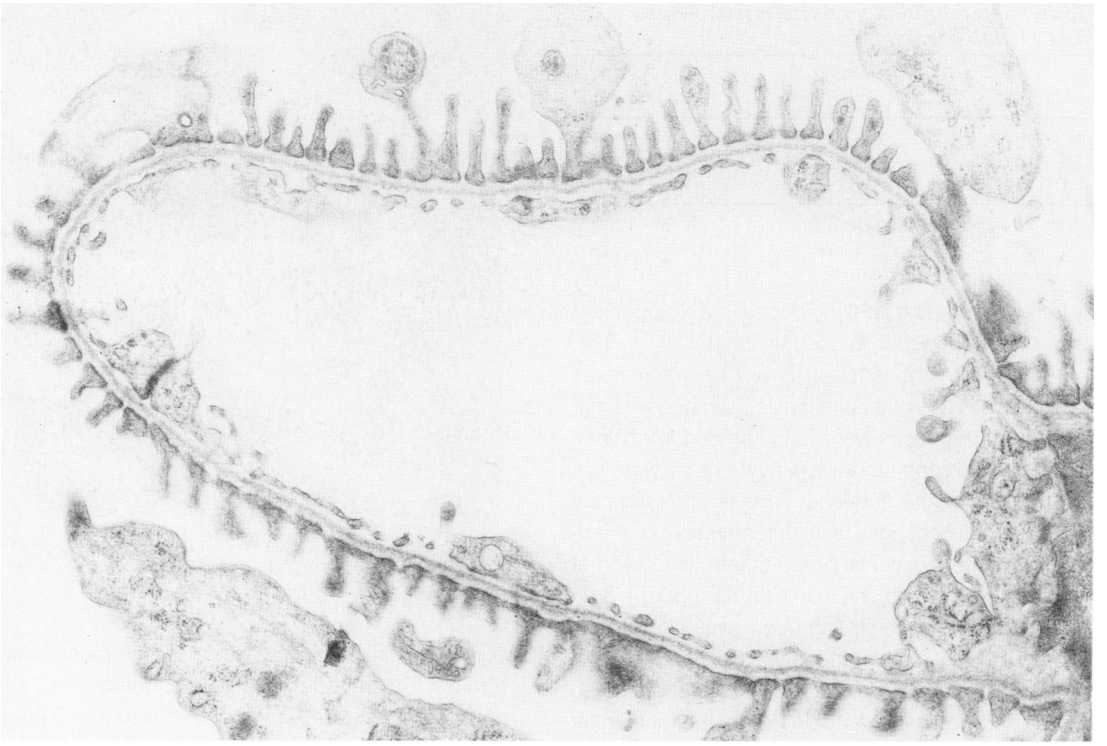

Figure 5. *Electron micrograph of glomerular capillary loop in an animal infused with vehicle for 5 hours. No ultrastructural changes can be detected (×7000).*

tached from the GBM (Figure 8). Polymorphonuclear leukocyte infiltration in the glomerular capillary lumens was always a remarkable finding. In two of eight rabbits fibrin-like material was observed mainly as free strands in the glomerular capillary lumens (Figure 9). Occasional platelets were also detected in few capillary loops. Glomerular epithelial cells showed few limited areas of fusion of foot processes. In some peritubular capillaries the presence of polymorphonuclear leukocytes was displayed associated with a moderate swelling of endothelial cells. No significant damage was detected in tubules, interstitium, and arterial vessels. In the additional group of rabbits killed 15 hours after the infusion of 8.0 μg/kg/h TNF ended, few polymorphonuclear leukocytes were detected in the glomerular capillaries and the endothelial swelling was mild. In the animals killed 24 hours after the end of TNF infusion no ultrastructural changes could be seen in the glomeruli. Similar to light microscopy findings, the examination of animals treated with boiled TNF and 0.5 ng/kg/h of endotoxin showed no significant glomerular abnormality, whereas in rabbits infused with TNF treated with polymyxin B the typical pattern of endothelial damage and polymorphonuclear leukocytes accumulation was present. When TNF was infused in an isolated perfused kidney system the ultrastructural examination did not show any endothelial damage in the glomerular capillaries, demonstrating that TNF does not possess direct "toxic" effects on glomerular endothelial cells. In the group of animals treated with IL-1β alone, the endothelium was mildly swollen and some polymorphonuclear leukocytes and platelets were detected in the glomerular capillary lumens. The association of TNF and IL-1β induced a marked endothelial swelling and a diffuse loss of fenestrae. Polymorphonuclear leukocyte infiltration was remarkable and platelets were seen frequently in the glomerular capillary lumens. We were unable to detect fibrin deposits in the glomeruli, however.

Discussion

This study shows that a 5-hour continuous infusion in rabbits of TNF doses of 0.8 and 8.0 μg/kg/h induced anemia, leukopenia, thrombocytopenia, and glomerular damage. At the renal level, animals infused with TNF showed glomerular endothelial cell changes and polymorphonuclear cell accumulation in capillary lumens. Fibrin deposits at the glomerular level were observed with the highest dose of TNF. The highest dose of TNF also induced a significant rise in serum creatinine.

To our knowledge this is the first report of glomerular damage induced by TNF. In 1986 Tracey et al,[14] who in-

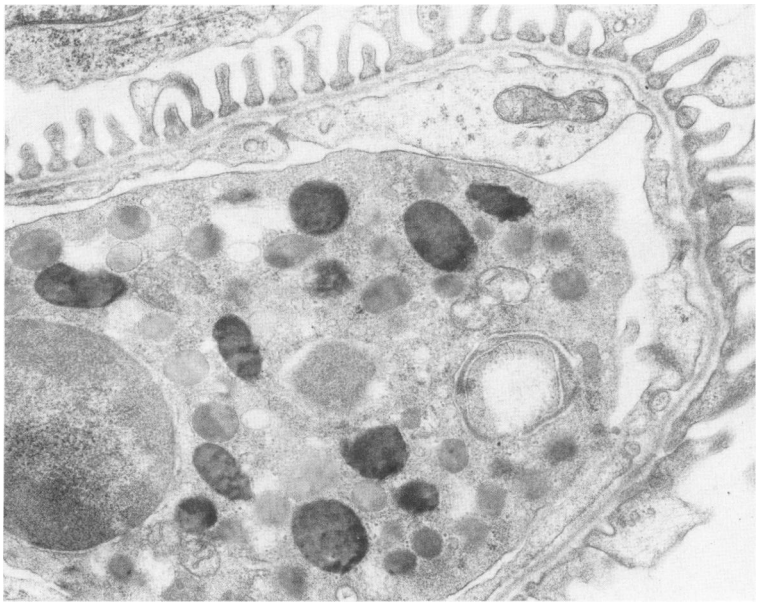

Figure 6. *Electron micrograph of a glomerular capillary loop in a rabbit infused with 0.8 μg/kg/h TNF for 5 hours. Note a mild endothelial swelling and the presence of a polymorphonuclear leukocyte in the glomerular lumen (×12,000).*

fused purified TNF to Sprague-Dawley rats at a dose considerably higher than that employed in the present investigation, found diffuse pulmonary inflammation and hemorrhage associated with ischemic and hemorrhagic lesions of the gastrointestinal tract. At the renal level the predominant finding was tubular necrosis. No renal lesion has been reported in mice when lower doses of TNF were employed.[25] More recently Gresser et al[26] reported inhibition of growth, severe fatty acid changes, and liver necrosis in suckling mice given TNF as a daily injection. No specific renal lesions have been detected but protein droplets were found in proximal tubular cells.

The present study found that endothelial cell damage and accumulation of polymorphonuclear cells in the glomerular capillary lumens were the main renal morphologic abnormalities associated with TNF infusion in the rabbit. Moreover, the accumulation of polymorphonuclear cells in the glomerular capillary lumens was associated with a fall in peripheral leukocyte count. These data are consistent with the finding that, even at very low concentrations, TNF can impair endothelial cell function[27] and has potent chemotactic activity for inflammatory cells.[28] It has been found that TNF enhances endothelial cell procoagulant activity[27,29] and suppresses the activity of an endothelial

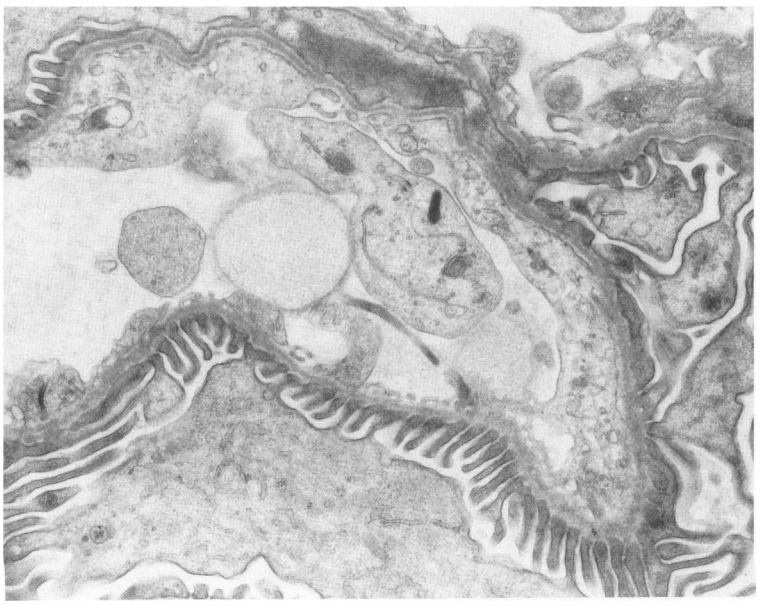

Figure 7. *Electron micrograph of a glomerular capillary loop in a rabbit infused with 8.0 μg/kg/h TNF for 5 hours. Note the presence of a marked swelling of glomerular endothelium. An area of fusion of foot processes is also present (×7000).*

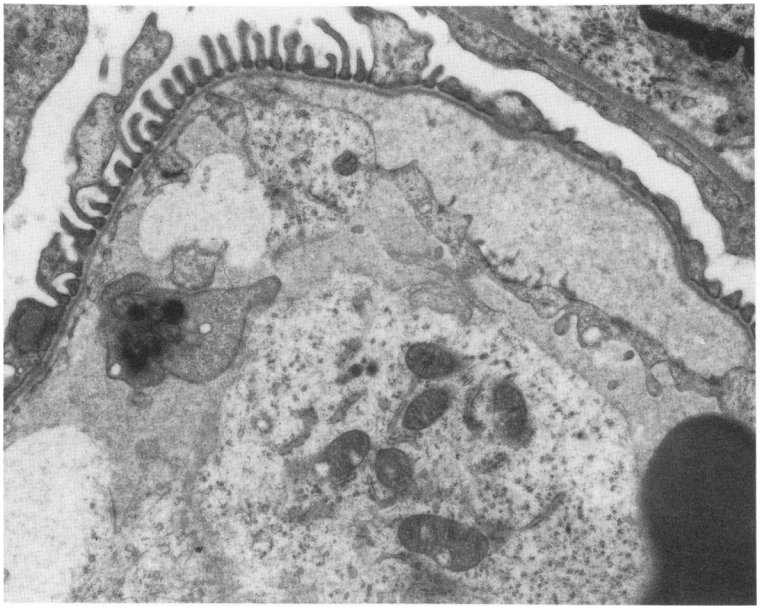

Figure 8. *Electron micrograph of a glomerular capillary in a rabbit infused with 8.0 μg/kg/h TNF. The endothelium is swollen and detached from the glomerular basement membrane. In the narrowed lumen a platelet is present (×7000).*

cell cofactor necessary for anticoagulant protein C pathway.[27] It also has been shown that TNF enhances the adherence of neutrophils to endothelial cells through mechanisms inducing the regulation of expression of cell surface molecules both on neutrophils and vascular endothelial cells.[30]

To address whether endothelial damage is the cause or consequence of leukocyte accumulation in the glomer-

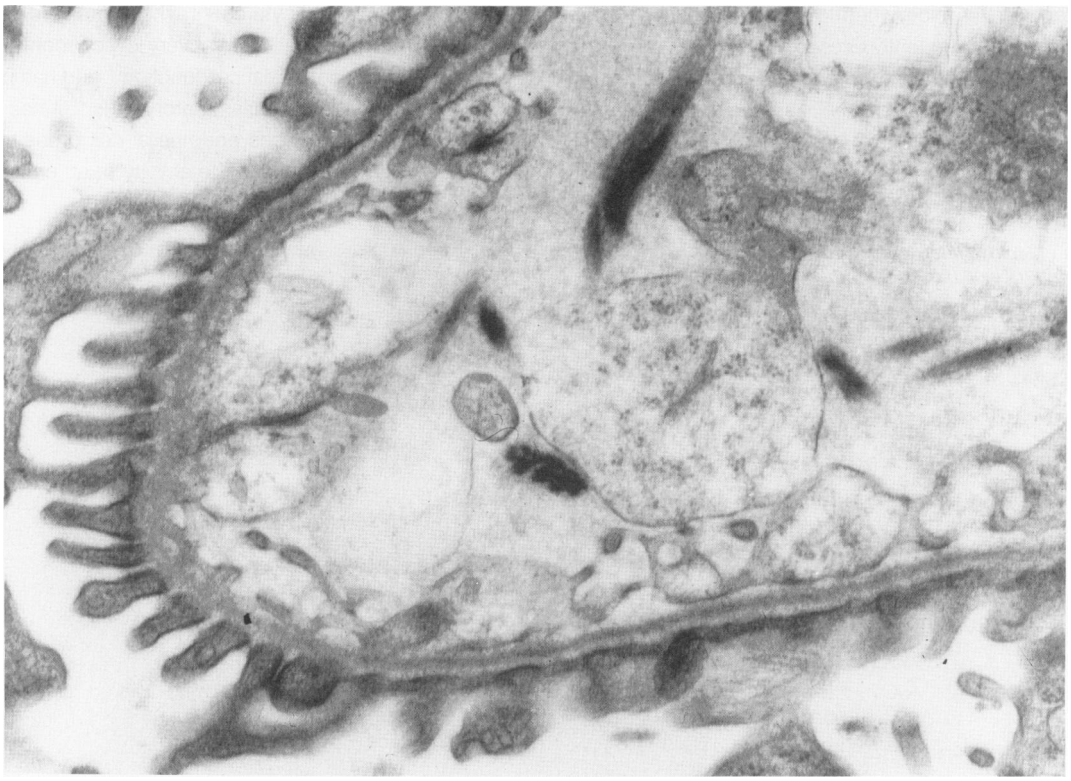

Figure 9. *Electron micrograph of a glomerular capillary in a rabbit infused with 8.0 μg/kg/h TNF displaying a swollen endothelium and some fibrin strands (×12,000).*

uli of TNF-treated animals we have used a system of *in situ* isolated rat kidney perfused with a cell-free medium containing different concentrations of TNF. Failure to find signs of endothelial damage in these preparations suggests that neutrophil and possibly platelet activation are crucial to the damaging effect of TNF on glomerular structure. Thus, TNF, by virtue of its effect on circulating inflammatory cells, may in turn induce endothelial cell activation that alters the normal homeostasis at the blood vessel wall interface and favors fibrin formation in glomerular capillary lumens. That this can be true is supported by the finding of fibrin deposits at glomerular level in animals injected with 8.0 μg/kg/h TNF for 5 hours.

Rabbits injected with the highest dose of TNF showed a significant rise in serum creatinine, a possible marker of renal insufficiency. However, these animals had only mild tubulointerstitial or vascular changes at renal level, which made it difficult to interpret the renal functional changes purely on the basis of the glomerular abnormalities. A possible interpretation of these findings is that mediators released from activated neutrophils that accumulate within glomerular capillaries participate to the observed decrease in the glomerular filtration rate in animals given TNF. This interpretation is consistent with recent data showing that selective receptor antagonists for thromboxane A2 and leukotrienes prevent the early reduction of perfusion and filtration in response to endotoxin in rats as evaluated by micropuncture studies.[31]

Because TNF promotes arachidonic acid metabolism, inducing the formation of prostaglandins in cultured cells,[32] and cyclooxygenase inhibitors block most of the *in vivo* toxic effects of TNF,[33] the possibility that the reported effects of TNF are mediated by eicosanoid synthesis must be considered. To evaluate this possibility we have studied the glomerular generation of TxB2, the stable breakdown product of TxA2, and 6-keto-PGF1α, the stable derivative of PGI2, in animals infused with vehicle or TNF at the doses of 0.8 and 8.0 μg/kg/h for 5 hours and killed at the end of the infusion. Failure to show any significant change in glomerular eicosanoid generation in animals infused with TNF in respect to animals infused with the vehicle would indicate that the effect of TNF on the glomerular structure is not mediated by arachidonate metabolites. Another possibility is that the effects of TNF are mediated by endogenous IL-1 because TNF induces IL-1 *in vitro* and *in vivo*.[34] To verify this possibility we performed selected experiments infusing either IL-1 alone or IL-1 plus TNF and compared the degree of glomerular inflammation with that found after TNF alone. The results indicated that IL-1 is significantly less potent than TNF in promoting glomerular inflammation whereas it appears to potentiate the effect of TNF. Thus, IL-1 is unlikely to be the major mediator of the glomerular inflammatory reaction that follows the infusion of TNF to the rabbit.

In summary, these results indicate that TNF can induce glomerular damage and favor fibrin formation in glomerular capillary lumens. These findings support the recent hypothesis[15] that the tissue injury elicited by endotoxin may be mediated by TNF and suggest new perspectives for the treatment of syndromes of microvascular thrombosis with maneuvers aimed at reducing the generation of TNF. Based on the present findings, TNF can now be regarded as a possible mediator of damage in renal diseases.

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