TNF-α Increases Albumin Permeability of Isolated Rat Glomeruli Through the Generation of Superoxide

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Abstract. Tumor necrosis factor-α (TNF-α) is a cytokine that plays a central role in inflammation. Glomerular levels of TNF-α are elevated in human and experimental glomerulonephritis. Glomerular cells produce and respond to TNF-α. One of the mechanisms by which these cells respond to TNF-α is through generation of reactive oxygen species. In this study, the effect of TNF-α on albumin permeability ($P_{albumin}$) of isolated rat glomeruli and the possible mechanism of this effect were examined. Isolated rat glomeruli were incubated with TNF-α (0.4 ng/ml), TNF-α with anti-TNF-α antibodies, and TNF-α with the reactive oxygen species scavengers superoxide dismutase, catalase, DMSO, or dimethylthiourea for 12 min at 37°C, and $P_{albumin}$ was calculated. TNF-α increased $P_{albumin}$ of isolated glomeruli compared with control (0.70 ± 0.02, $n = 25$ *versus* 0.00 ± 0.05, $n = 26$), and this effect was abrogated by anti-TNF-α antibodies (−0.18 ± 0.05, $n = 23$). Superoxide dismutase abolished the increase in $P_{albumin}$ (−0.04 ± 0.11, $n = 23$), whereas catalase (0.73 ± 0.08, $n = 30$), DMSO (0.64 ± 0.03, $n = 10$), or dimethylthiourea (0.51 ± 0.08, $n = 10$) did not alter the effect of TNF-α. These results indicate that TNF-α increased $P_{albumin}$ of isolated glomeruli and that the mediator of the increased $P_{albumin}$ is superoxide. It is concluded that TNF-α derived from glomerular or extraglomerular sources can increase glomerular $P_{albumin}$ through generation of superoxide and may lead to proteinuria. (J Am Soc Nephrol 9: 433–438, 1998)

Tumor necrosis factor-α (TNF-α) is a 17-kD cytokine that plays a central role in both systemic and glomerular inflammation and injury (1,2). Proteinuria is a common finding in inflammatory states and reflects dysfunction of the glomerular permeability barrier (3). Several systemic conditions are characterized by elevated serum or plasma levels of TNF-α, including AIDS, malignancy, chronic infection, and sepsis (4–8). Although TNF-α is produced mainly by macrophages and monocytes, other cells, including mesangial cells and glomerular epithelial cells, are capable of TNF-α production (9,10). Renal and glomerular levels of TNF-α are elevated in animal models of glomerular disease and human glomerulonephritis (10–15). Additionally, TNF-α levels were found to be elevated in the serum and urine of patients with nephrotic syndrome due to focal segmental glomerulosclerosis (16). TNF-α induces the production of several inflammatory mediators and enzymes by mesangial cells and glomerular epithelial cells, including reactive oxygen species (ROS), eicosanoids, and other cytokines (17–21). Some of these mediators are known to alter the glomerular capillary permeability barrier.

We have developed an *in vitro* method to study the effect of various inflammatory mediators on glomerular albumin permeability ($P_{albumin}$), using isolated glomeruli (22). With this method, we studied the direct effect of TNF-α on $P_{albumin}$ and identified a possible mediator of this effect.

Materials and Methods

Experimental Animals

Normal male Sprague Dawley rats (180 to 250 g body wt) maintained on Purina chow and water *ad libitum* were used in all experiments. All animal experimentation was conducted in accord with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Medium

Isolation and washing medium contained (in mmol/L): 108 sodium chloride, 2.5 potassium phosphate; 25 sodium bicarbonate, 1.2 magnesium sulfate, 2.0 calcium chloride, 1.0 sodium citrate, 4.0 sodium lactate, 6.0 L-alanine, and 5.3 glucose. The oncotic content of the medium was due to 4 g/dl bovine serum albumin (BSA) in isolation and incubation medium. The washing medium contained 1 g/dl BSA. The oncotic pressure of isolation and washing media was measured using a membrane oncometer (model 4100; Wescor, Logan, UT). The pH of the medium was adjusted to 7.4 before use.

ROS Scavengers and Inhibitors

All chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO). The concentration of the various scavengers and inhibitors used is: 250 U/ml superoxide dismutase (SOD), 25 μg/ml catalase, 1.0 mM DMSO, and 1.0 mM dimethylthiourea (DMTU).

Received July 14, 1997. Accepted September 26, 1997.

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1046-6673/0903-0433$03.00/0
Journal of the American Society of Nephrology

TNF-α and Antibodies

Recombinant human TNF-α was kindly supplied by Dr. Paul Terranova of the University of Kansas Medical Center. The bioactivity of the recombinant human TNF-α was 6.27×10^7 U/mg. The concentration used in most experiments was 0.4 ng/ml, which is within the physiologic range of the cytokine. In some experiments, other concentrations were used, *i.e.*, 1.0, 0.2, 0.1, or 0.02 ng/ml. Rabbit polyclonal antihuman TNF-α antibodies (titer 1.2 million, determined by enzyme-linked immunoassay) were also supplied by Dr. Terranova, and were used in a vol/vol mix with TNF-α. Sera from nonimmunized rabbits were used as control.

Isolation of Glomeruli

Sprague Dawley rats were anesthetized with metaphane, and kidneys were removed aseptically. Glomeruli from the outer 1 to 2 mm of renal cortex were isolated in medium containing 4 g/dl BSA, using standard sieving techniques as described previously (23). This method of preparation yields glomeruli largely free (>95%) of Bowman's capsule. Using this technique, fewer than two cells/glomerulus fail to exclude trypan blue.

Incubation of Glomeruli

Experimental treatments were added to isolated glomeruli, which were then immediately incubated at 37°C for 12 min. In those studies in which multiple reagents were used, these were added simultaneously. Control glomeruli were incubated with 2% vol/vol normal human serum.

Measurement of Glomerular Volume Change

Isolated glomeruli were allowed to adhere to coverslips coated with poly-L-lysine (1 mg/ml) for 10 to 15 s; unattached glomeruli were swept away by gentle washing with fresh isolation medium. Adherent glomeruli were observed and selected for their initial images using videomicroscopy. All selected glomeruli were free of Bowman's capsule. After an initial period of observation, the medium was replaced with washing medium of lower oncotic pressure. Volume changes in glomeruli subsequent to the applied oncotic gradient occurred within 5 s and were maintained for at least several minutes in both control and experimental conditions. Repeat images were obtained 2 to 3 min after a change in medium. Initial and final volumes of each glomerulus were calculated from the average diameter measured on the video monitor. Volume change (ΔV) was calculated as:

$$\frac{(V_{final} - V_{initial})}{V_{initial}} \times 100.$$

At least five glomeruli from each of three to five rats were studied in each experimental condition.

Use of Volume Change to Calculate $\sigma_{albumin}$

The increase in glomerular volume after changes in bathing media is a complex function that depends on the relative exchangeable volume of the glomerulus, the spatial arrangement of the capillaries, and the compliance and elasticity of the capillary wall, as well as on the permeability characteristics of the filtration barrier.

The rationale and calculations for the measurement of $\sigma_{albumin}$, using the relationship between ΔV and the oncotic gradient ($\Delta 80$) of control and experimental glomeruli, have been described in our earlier studies (22). Isolated, nonperfused glomeruli exhibit a volumetric response to oncotic gradients. We have shown previously that there is a direct relationship between the increase in glomerular volume (ΔV) and the oncotic gradient ($\Delta 80$) applied across the capillary wall. The slope of this relationship is determined by the reflection coefficient of the solute used. We used this principle to calculate reflection coefficient of albumin ($\sigma_{albumin}$), using the ratio of ΔV of experimental to control glomeruli in response to identical albumin gradients. This calculation is possible because $\sigma_{albumin}$ of control glomeruli is equal to 1.0. Glomerular volume was measured before and within 2 to 3 min after replacing isolation medium containing 4 g/dl BSA, with washing medium containing 1 g/dl BSA.

$$\sigma_{albumin} = (\Delta V_{experimental}/\Delta V_{control}).$$

Convectional Permeability

Convectional permeability of albumin ($P_{albumin}$) was defined as:

$$P_{albumin} = (1 - \sigma_{albumin}).$$

When $\sigma_{albumin}$ is zero, $P_{albumin}$ is 1.0. When $\sigma_{albumin}$ is 1.0, albumin cannot cross the capillary and $P_{albumin}$ is zero.

Results

As shown in Figure 1, the $P_{albumin}$ of glomeruli incubated with TNF-α (0.4 ng/ml) increased significantly (0.70 ± 0.02, $n = 25$) compared with control glomeruli (0.00 ± 0.05, $n = 26$). The effect on $P_{albumin}$ was seen at doses as low as 0.2 ng/ml. Coincubation of glomeruli with TNF-α and anti-TNF-α antibodies abolished this effect of TNF-α (-0.18 ± 0.05, $n = 23$).

Additional experiments were carried out to determine if ROS were involved in the TNF-α-mediated increase in $P_{albumin}$. Isolated glomeruli were incubated with TNF-α or with TNF-α and a specific ROS scavenger. As shown in Figure 2, SOD, a scavenger of superoxide, abolished the effect of TNF-α on $P_{albumin}$ (-0.04 ± 0.11, $n = 23$). Superoxide alone had no effect on albumin permeability (0.06 ± 0.02, $n = 22$).

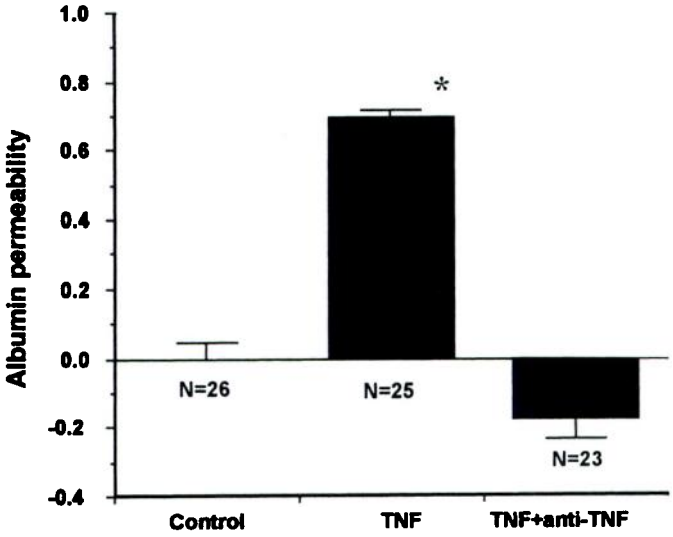

Figure 1. Effect of tumor necrosis factor-α (TNF-α) and anti-TNF-α antibodies on albumin permeability. Albumin permeability of isolated rat glomeruli is significantly increased after incubation with TNF-α. This effect is prevented by coincubation with anti-TNF-α antibodies. *$P < 0.01$ compared with control.

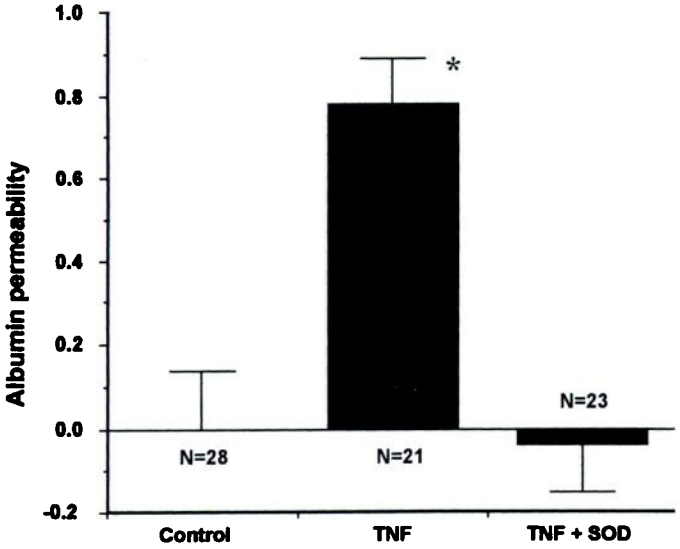

Figure 2. Effect of superoxide dismutase (SOD) on TNF-α-mediated increase in albumin permeability. Addition of SOD to the incubation medium abolished the increase in albumin permeability caused by TNF-α. SOD itself has no effect on albumin permeability. $*P < 0.01$ compared with control.

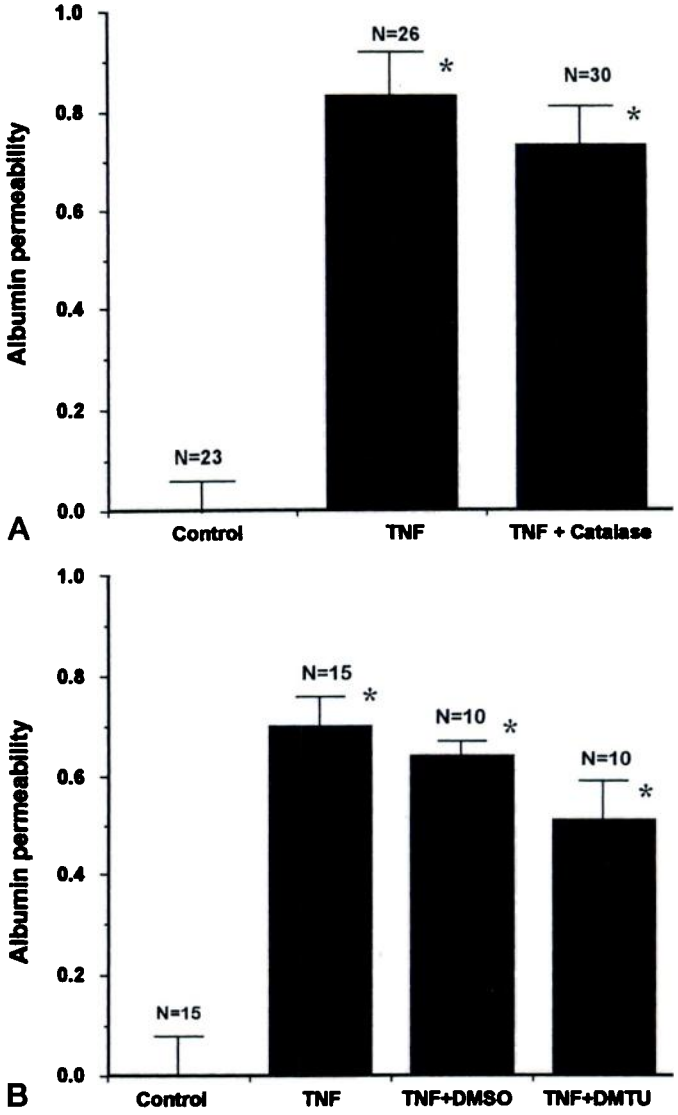

Figure 3. Effect of reactive oxygen species scavengers on TNF-α-mediated increase in albumin permeability. $*P < 0.01$ compared with control. (A) Addition of catalase does not alter the effect of TNF-α on albumin permeability. Catalase itself has no effect on albumin permeability. (B) Addition of DMSO or dimethylthiourea (DMTU) does not alter the effect of TNF-α on albumin permeability. Neither DMSO nor DMTU has an effect on albumin permeability.

Catalase, a scavenger of hydrogen peroxide, had no effect on the increase in $P_{albumin}$ caused by TNF-α (0.73 ± 0.08, $n = 30$) (Figure 3A). Similarly, neither DMSO nor DMTU, both scavengers of hydroxyl radical, prevented the increase in $P_{albumin}$ (0.64 ± 0.03, $n = 10$, and 0.51 ± 0.08, $n = 10$, respectively) (Figure 3B). Scavengers alone failed to alter the $P_{albumin}$ (catalase: 0.17 ± 0.11, $n = 27$; DMSO: 0.25 ± 0.07, $n = 14$; DMTU: 0.17 ± 0.07, $n = 15$).

Discussion

We have demonstrated a direct effect of TNF-α on the protein permeability barrier of the glomerulus that is independent of alterations in hemodynamic factors or effects of recruited inflammatory cells. TNF-α significantly increased the $P_{albumin}$ of isolated rat glomeruli in this study. Coincubation of glomeruli with anti-TNF-α antibodies abolished this effect, indicating that the effect was specific to TNF-α. Doses of TNF-α as low as 0.2 ng/ml significantly increased $P_{albumin}$. SOD, a scavenger of superoxide, abolished the TNF-α-mediated increase in $P_{albumin}$, whereas catalase, DMTU, or DMSO, scavengers of hydrogen peroxide or hydroxyl radical, respectively, did not alter the effect of TNF-α on $P_{albumin}$. These results implicate superoxide as an important mediator in the effect of TNF-α on $P_{albumin}$.

Proteinuria is a nonspecific manifestation of glomerular injury and is seen in systemic and renal diseases that are characterized by inflammation or elevated cytokine production (3). Cytokines mediate many of the manifestations of inflammation and disease (24). TNF-α, a 17-kD cytokine produced mainly by monocytes and macrophages, plays a central role in inflammation (1). Circulating levels of TNF-α are elevated in systemic diseases such as AIDS, malignancy, chronic infection, and sepsis (4–8). Additionally, TNF-α is an important mediator of glomerular dysfunction (2). Suranyi *et al.* showed that TNF-α levels in plasma and urine are elevated in patients with nephrotic syndrome due to focal segmental glomerulosclerosis or membranous nephropathy (16). Elevated renal or glomerular levels of TNF-α are seen in toxic serum nephritis (11), anti-glomerular basement membrane nephritis (12), lupus nephritis (13), acute renal failure of sepsis (14), and antineutrophil cytoplasmic antibody-positive glomerulonephritis (15). Glomeruli from rabbits treated with infusions of TNF-α show extensive glomerular damage on histologic examination (25). In addition to systemic sources, glomerular cells are potential sources of TNF-α. Glomeruli isolated after infusion of lipopolysaccharide (LPS) produced significant amounts of TNF-α, as did glomeruli exposed to LPS only after isolation (26). The

amount of TNF-α produced was not altered by prior irradiation of the animal, indicating that influx of bone marrow-derived cells was not required for this effect.

Mesangial cells in culture produce TNF-α after stimulation by LPS (27), doxorubicin, or puromycin aminonucleoside (PAN) (28), and release is modified by desferrioxamine (29). TNF-α was demonstrated in mesangial cells of patients with lupus nephritis (30). Glomerular epithelial cells in culture produce TNF-α in response to doxorubicin or PAN, and TNF-α is directly toxic to glomerular epithelial cells (10). Additionally, TNF-α was expressed by glomerular epithelial cells in biopsies of human membranous nephropathy and lupus membranous nephropathy (31).

TNF-α acts on glomerular cells in several ways. TNF-α stimulates glomerular epithelial cells in culture to produce various mediators, including plasminogen activator and inhibitor (32), gelatinase (33), and procoagulant tissue factor (34). TNF-α stimulates formation of cAMP (18) and cGMP (35) in mesangial cells in culture, and increases synthesis of prostaglandin E_2 (36) and platelet-activating factor (37). TNF-α causes the release of the ROS superoxide and hydrogen peroxide from cultured human mesangial cells in amounts comparable to those produced by activated macrophages (17).

There is ample evidence that ROS are crucial mediators in inflammatory and noninflammatory glomerular disease (38,39). Production of ROS is associated with increased albumin permeability in several animal models (40–46), and blocking the effects of these mediators with scavengers is associated with improvement of proteinuria (45–47). Wang *et al.* showed that treatment of rats with puromycin aminonucleoside (PAN) nephrosis with cyclosporin A decreased proteinuria; treated rats also showed higher activities of glomerular SOD and catalase and attenuation of foot process effacement (48). We have shown that superoxide generated by either xanthine/xanthine oxidase system or phorbol myristate acetate (PMA)-activated macrophages increases $P_{albumin}$ of isolated glomeruli, and this effect is abrogated by SOD but not catalase (49). We have also shown that incubation of isolated glomeruli with PMA-activated rat polymorphonuclear cells increased $P_{albumin}$ and that this increase is prevented by catalase, SOD, taurine, or sodium azide, implicating hypohalous acid in the effect on $P_{albumin}$ (50).

Resident glomerular cells are capable of ROS production. Glomerular epithelial cells in culture produce ROS in response to various toxins such as doxorubicin and PAN (51,52). Ricardo *et al.* showed that administration of SOD to rats with PAN nephrosis not only decreased proteinuria, but also protected podocyte foot processes as examined with electron microscopy (53). Mesangial cells in culture produce ROS in response to immune complexes (54), PMA (55), platelet-activating factor (56), and cytokines, including TNF-α (17).

We have developed an *in vitro* method to study glomerular capillary albumin permeability, using isolated glomeruli (22). This method is advantageous in that it allows us to test single variables and eliminates possible effects of hemodynamic changes and circulating cells or factors. Using this method, we were able to eliminate the many potential systemic inflammatory and hemodynamic effects of TNF-α and avoid possible glomerular damage by infiltrating inflammatory cells.

We observed an effect of TNF-α on albumin permeability after 12 min of incubation. Many investigators have noted that some effects of TNF-α on target cells can be seen quite quickly, sometimes within a matter of seconds (1). Examples of rapid effects of TNF-α include accumulation of cAMP in fibroblasts (57) and serine phosphorylation of cytosolic proteins in U937 cells (58). Radeke *et al.* (17) showed that mesangial cells in culture produce significant amounts of ROS after exposure to TNF-α, though no appreciable levels of ROS were seen before 50 min of incubation. Several factors could contribute to the difference in time course in these studies. Our study is a bioassay that depends on cellular response to experimental manipulations. Mediators such as superoxide may act in an autocrine or paracrine manner, rapidly achieving high levels in the local milieu. Glomerular cells may respond to very high local levels of mediators even if the concentration in the medium is below the detectable level. Radeke *et al.* used an assay of ROS in culture medium. Accumulation of ROS in medium may have been delayed compared with initial generation. Additionally, it is possible that mesangial cells in culture respond differently to TNF-α than glomerular cells *in situ*. Cells in the isolated glomerulus may be more sensitive to TNF-α and respond more rapidly.

The cellular or structural target of TNF-α action is not clear. Although endothelial cells may respond to TNF-α, the glomerular capillary endothelial cell is not thought to play a significant role in the protein permeability barrier. TNF-α may be affecting the glomerular basement membrane, either by alteration in the structural constituents or diminution of the negative charge of the glomerular basement membrane. It is unlikely that either of these is the mechanism of the change seen in such a short time period. TNF-α stimulates ROS production by cultured mesangial cells; the amounts of ROS generated are comparable to those produced by activated macrophages. It is possible that the TNF-α in our system stimulates mesangial cells of isolated glomeruli to produce superoxide, which in turn alters the permeability barrier by affecting the structure and/or function of one or more components of the barrier. The glomerular epithelial cell is thought to play a significant role in maintenance of the permeability barrier; thus, changes in the function of this cell type could lead to alterations in $P_{albumin}$. Glomerular epithelial cells are capable of ROS production in response to various stimuli (51,52). ROS may alter the properties of the glomerular epithelial cell membrane, cytoskeleton, and/or intercellular junctions possibly by lipid peroxidation, or may induce the production of other mediators such as eicosanoids, cyclic nucleotides, or cytokines, leading to increased $P_{albumin}$ in the experimental situation.

We have shown that TNF-α has a direct effect on the glomerular protein permeability barrier, and that the ROS superoxide may play an important role in the mediation of this effect. Such effects on the filtration barrier by TNF-α through a ROS mediator may explain proteinuria seen in clinical settings characterized by increased circulating or glomerular TNF-α.

Acknowledgments

This work was supported by U.S. Public Health Service Grant 22040, a Biomedical Research Support Grant from National Institutes of Health (2 S07 RR 05373), and by grants from the Kansas Affiliate of the American Heart Association.

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