Cytokine Formation Within Rat Glomeruli During Experimental Endotoxemia

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ABSTRACT

Increasing evidence supports a role of cytokines, tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), and IL-6 in the development of endotoxin-induced acute renal failure. Several activities of these cytokines require a local rather than a systemic production and function. Thus, this study investigates the chronology of cytokine expression in glomeruli isolated from normal rats or rats given IV lipopolysaccharide injections. Detectable levels of TNFα could be found in glomeruli isolated from normal rats as assessed by L-929 fibroblast lytic assay and ELISA. Glomeruli isolated from rats given lipopolysaccharide transiently released increased amounts of TNFα in relation to the dose of lipopolysaccharide (10 to 500 µg/kg body wt) and the lag period between lipopolysaccharide injection and glomerular isolation (20 to 120 min). TNFα was released in similar amounts by glomeruli from normal rats that were exposed in vitro to lipopolysaccharide challenge (0.01 to 10 µg/mL), indicating that lipopolysaccharide had direct effects on the release of TNFα from glomerular cells. These cells consisted mainly of resident cells because reduction of glomerular infiltration by bone marrow–derived cells after the irradiation of normal rats did not affect TNFα release. Glomerular IL-1 and IL-6 production was evaluated by specific bioassays under identical conditions. No IL-1 activity could be detected in the medium or within the glomerular cells at any time within 120 min after lipopolysaccharide injection. By contrast, glomerular IL-6 production was induced after lipopolysaccharide challenge both in vivo and in vitro. Specific neutralization of TNFα did not affect IL-6 production, whereas neutralization of IL-6 slightly enhanced TNFα release. Thus, these results suggest that in endotoxin-induced acute renal failure: (1) TNFα and IL-6 are produced by glomeruli in response to lipopolysaccharide, and (2) TNFα may possibly contribute to the development of glomerular hemodynamic changes as suggested by its time course of production.

Key Words: Acute renal failure, lipopolysaccharide, glomeruli, tumor necrosis factor alpha, interleukins

Severe infections, mainly with gram-negative bacteria, can result in organ dysfunctions, including acute oliguric renal failure. Even in the absence of systemic hypotension, release of endotoxin lipopolysaccharide (LPS) from the cell wall of these bacteria leads to an increase in renal vascular resistance and a decrease in GFR (1). The pathogenic mechanisms responsible for these changes involve a large variety of mediators, such as angiotensin II, adenosine, catecholamines, eicosanoids, and platelet-activating factor (PAF) (2–5). A recent series of investigations has suggested that these metabolites are the final mediators of endotoxic shock, whereas cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1) 1, and IL-6, are early mediators. Three main lines of evidence indicate that TNFα plays a prominent role: (1) high circulating levels of TNFα occur in animals given injections of LPS or in patients with septic shock due to gram-negative bacteremia (6). (2) injection of recombinant TNFα induces the pathologic changes of endotoxic shock (7), and (3) administration of neutralizing anti-TNFα antibodies prevents these changes (8). Because TNFα is known to induce in vitro mesangial cell contraction (9) and increased mesangial production of adenosine (10), eicosanoids (11), and PAF (9), it may indeed contribute to the fall of GFR during endotoxia.

Blood-borne cells, both blood monocytes and tissue macrophages, are a major source of TNFα (12). Whereas the systemic production of TNFα in experimental endotoxemia has been documented (6), there has been little evidence for its local production within the kidney. Recently, Ulich et al. (13) and Heinzel (14) have shown that TNFα messenger RNA is present in increased quantities in the kidney several
minutes after LPS injection in the rat and in the rabbit, respectively. The recruitment of activated blood mononuclear cells to the glomerulus and the activation of both resident glomerular (15, 16) and tubular cells (17) may contribute to this expression. In this study, we sought to determine the production of TNFα by glomerular cells in a model of LPS-induced acute renal failure in the rat and its relationship to the production of IL-1 and IL-6. We show that detectable amounts of TNFα and IL-6, but not of IL-1, are produced by isolated glomeruli early after in vivo or in vitro exposure to LPS.

MATERIALS AND METHODS

Induction of Endotoxemic Acute Renal Failure and Isolation of Rat Glomeruli

Experiments were performed on male Sprague-Dawley rats (110 to 220 g) receiving normal chow and water ad libitum. Septic shock was induced by a single iv injection of LPS (from Escherichia coli 026:B6; Sigma Chemical Co., St. Louis, MO) diluted in 0.16 M NaCl. The injected dose varied from 10 to 500 μg/kg body wt. At 20 to 120 min postinjection, rats were anesthetized with pentobarbital (60 mg/kg body wt ip). Blood samples were collected for TNFα assay, and kidneys were removed after an aortic perfusion with 50 mL of sterile 0.16 M NaCl. The kidneys were decapsulated, and the cortex was minced and successively pressed through 106- and 75-μm-pore-size sieves, as previously described (18). The glomeruli retained on the 75-μm-pore-size sieve were harvested and rinsed in ice-cold Dulbecco’s phosphate-buffered saline. They were suspended in RPMI 1640 medium (Flow Laboratories, Irvine, United Kingdom) without fetal calf serum (FCS) and were then counted. Glomerular preparations assessed under light microscopy exhibited less than 5% tubular contamination.

Incubation of Glomeruli

Glomeruli isolated from rats given LPS were suspended in RPMI 1640 medium without FCS, seeded in 24-well plates (Nunc, Roskilde, Denmark) [10,000 glomeruli/300 μL/well], and incubated for 1 to 6 h in a 37°C humid atmosphere with 5% carbon dioxide. Glomeruli isolated from control and X-irradiated rats were suspended in RPMI 1640 medium supplemented with 1% FCS, seeded in 24-well plates, and incubated for 1 to 24 h with serial dilutions of LPS. In some cases, glomeruli were incubated for 4 h with an anti-murine TNFα neutralizing antibody (10⁶ neutralizing units/mL; Genzyme, Cambridge MA) diluted at 1/100, with an anti-human IL-6 monoclonal antibody provided by Dr. D. De Croote (Medgenix, Fleurus, Belgium), or with recombinant mouse IL-6 (5 × 10⁵ U/mL; Genzyme) diluted at 1/5. After the incubation, the media were harvested and centrifuged. The supernatants were then frozen at −70°C for cytokine analysis.

TNFα Assays

TNFα content in the supernatants of isolated glomeruli and serum samples was assayed by both TNFα bioassay and TNFα ELISA. The biologic activity of TNFα was determined by the measurement of the cytotoxicity of serum samples and supernatants on the fibrosarcoma murine cell line L-929 (16). Briefly, the L-929 target cells were radiolabeled with 51Cr ([51Cr]sodium chromate, 5 μCi/mL; the Radiochemical Centre, Amersham, United Kingdom) and were then incubated at 37°C in 96-well microplates (3.10⁶ cells/well); each well contained 100 μL of modified Eagle medium (Boehringer, Mannheim, Germany) supplemented with 10% FCS, 1% nonessential amino acids (Flow), and 1% L-glutamine. After 18 to 24 h, the cells were washed twice with the same medium and incubated with 0.1 mL of modified Eagle medium containing 1 μg/mL of actinomycin D (Sigma) in the presence of serial dilutions of test supernatant or serum. In some cases, the supernatants were preincubated for 20 min at 37°C with an anti-murine TNFα neutralizing antibody (10⁶ neutralizing units/mL; Genzyme) diluted at 1/100. After overnight incubation at 37°C, the radioactivity was determined in both the cell-free supernatants and the adherent cells, which were lysed with 0.1 mL of 1 M NaOH. The cytotoxicity percentage was calculated by the following formula: %specific cytotoxicity = (R - S) × 100/(T - S) with R = cpm released in the presence of agonist, S = cpm spontaneously released, and T = total cpm.

The immunoreactive rat TNFα was measured by an ELISA (Genzyme) according to the conditions recommended by the supplier.

LAF Assay

IL-1 activity was determined by the standard thymocyte proliferation assay (19). Briefly, 10⁷ thymocytes obtained from LPS-low responder C3H/HeJ mice at 4 to 6 wk of age (CSEAL, Orleans, France) were incubated in 100 μL of RPMI 1640 medium containing 10% fetal bovine serum (FCS) 25 mM N-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 IU/mL of penicillin (Gibco), 100 μg/mL of streptomycin (Gibco), 50 μM 2-mercaptoethanol, 1 μg/mL of phytohemagglutinin (Wellcome Research Laboratories, Beckenham, England), and test samples (100 μL). Each determination was performed in duplicate. DNA synthesis was assayed for the last 8 h of a 72-h incubation at 37°C under a 5% CO₂ atmosphere by pulsing the cultures with 1 μCi/well of [3H]thymidine (5 Ci/mmol; CEA,
Saclay, France). In order to detect intracellular IL-1 activity, glomeruli were sonicated and centrifuged and the supernatant was assayed as described. Units of IL-1 activity were calculated by the following equation: IL-1 (units/milliliter) = cpm of test samples - background cpm/background cpm × reciprocal dilution of test sample. Background cpm is ³H-thymidine incorporated in thymocytes cultured in medium with phytohemagglutinin alone. To establish the sensitivity of the lymphocyte activating factor (LAF) assay, IL-1 activity was determined in peritoneal macrophages under the same conditions. Macrophages were elicited in rats by the ip injection of thioglycolate 5 days before peritoneal cells were harvested. Cells were adhered for 2 h and then challenged with 1 µg/mL of LPS for 18 h.

IL-6 Activity Assay

IL-6 activity was measured by the capacity of this cytokine to induce the proliferation of a sensitive B9 cell line (provided by Dr. L. Aarden, Central Laboratory of the Netherlands Red Cross Transfusion Service, Amsterdam, Netherlands) (20). The B9 cell line was maintained in Iscove medium (Biochrom KG, Berlin, Germany) supplemented with 2 mM L-glutamine, penicillin (100 IU/mL), streptomycin (100 µg/mL), 5% heat-inactivated FCS (referred to hereafter as culture medium), and a P388D1-conditioned medium as a source of IL-6 (provided by Dr. M. Dy, CNRS URA 1461, Hôpital Necker, Paris, France). After three washes to eliminate traces of remaining IL-6, B9 cells were resuspended in culture medium and further incubated at a density of 5,000 cells/50 µL/well of 96-well, flat-bottomed culture plates (Falcon 3072; Becton-Dickinson and Co, Lincoln Park NJ) with 50 µL of serial dilutions of duplicate supernatant samples for 72 h. Cellular proliferation was measured by the MTT (dimethyl-thiazol-2-yl diphenyltetrazolium bromide: Sigma) colorimetric assay at 570 nm with a microplate reader (MR 5000: Dynatech, Alexandria, VA) with a reference filter at 630 nm. Recombinant human IL-6 (Janssen Biochemica, Beere, Belgium), titrated along with the unknown supernatant samples, was diluted in 1% heat-inactivated FCS and tested as internal positive control. One IL-6 unit was defined as the concentration that leads to half-maximal B9 cell proliferation.

X-Irradiation Protocol

Rats were anesthetized and irradiated as described (21) so as to receive a total dose of 900 rads with a lead cuff protecting the kidneys. Four days later, the kidneys were removed and the glomeruli were incubated for 4 h with serial dilutions of LPS as described above. The depletion of glomerular Ia-positive phagocytes of extrarenal origin after whole-body irradiation has been evidenced. First, by light microscopy, blood-borne cells were found to be absent from capillary lumen. Second, by immunohistochemical technique with mouse monoclonal OX-6 antibody (SeraLab, Oxford, England), the number of Ia-positive cells was found to be low in the control group (1.56 ± 0.35 cells/glomerular section) and significantly reduced after whole-body irradiation (0.78 ± 0.18 cells/glomerular section; P = 0.05).

Statistical Analysis

Results are given as the mean ± standard error. The statistical significance of differences between experiments was analyzed by unpaired t tests or analysis of variance (ANOVA). A P value ≤ 0.05 was considered to be significant.

RESULTS

Characterization of TNFα Production by Glomeruli During Experimental Endotoxemia

The production of TNFα by glomeruli isolated from control rats was just at the limit of detection of the L-929 fibroblast lytic assay (Figure 1A). The production of TNFα by glomeruli isolated from kidney cortices 40 min after an iv injection of 0.2 mg/kg of LPS was significantly increased (P < 0.01). This activity fell toward baseline by 2 h. By comparison, analysis of serum TNFα bioactivity revealed a slightly delayed time course (Figure 1B). In subsequent experiments, glomeruli have been isolated 1 h after the LPS injection. The dose range of LPS resulting in increased glomerular production of TNFα was determined under these conditions (Figure 2). As little as 0.01 mg/kg of LPS enhanced glomerular TNFα production. Maximum increase occurred between 0.2 and 0.5 mg/kg of LPS.

The specificity of the assay for TNFα was confirmed by neutralization of the samples with an anti-mouse recombinant TNFα polyclonal antibody. The cytotoxic activity of glomerular supernatants was completely suppressed by the addition of this antibody at a dilution of 1/100, whereas the preimmune serum at the same dilution had no effect (data not shown). Moreover, a quantitative ELISA was used to measure the amount of immunoreactive TNFα produced by isolated glomeruli (Figure 3). Glomeruli isolated from control rats produced detectable amounts of TNFα (62.5 ± 36.6 pg/mL; mean ± SE; N = 3). This production increased progressively after an iv injection of LPS, reaching a peak by 1 h. After 1-h, the levels of immunoreactive TNFα remained practically unchanged, whereas TNFα bioactivity rapidly declined.
Glomerular Cytokines in Experimental Endotoxemia

**Figure 1.** (A) TNFα production by glomeruli isolated from rat kidneys at various periods of time after LPS injection (200 μg/kg). Glomeruli were seeded at 10,000/well and incubated at 37°C for 4 h. (B) Serum TNFα levels at various periods of time after LPS injection. Glomerular supernatants and serum samples were assayed for the presence of TNFα by bioassay. Results represented mean ± SE of five experiments. ANOVA indicated that TNFα cytotoxic activity varied significantly depending upon the time (P < 0.005).

**In Vitro Effects of LPS on Glomerular TNFα Production**

To determine whether glomerular cells respond directly to LPS or indirectly to substances released from LPS-activated tissues outside of the glomerulus, the production of TNFα by glomeruli isolated from control rats was measured after *in vitro* LPS challenge (Figure 4). The induction of bioactive TNFα release was observable with as little as 0.1 μg/mL of LPS and was increased in a dose-dependent manner. The simultaneous addition of anti-mouse recombinant TNFα polyclonal antibody resulted in the suppression of the LPS-induced cytotoxic activity.

Because TNFα could be produced by glomerular macrophages, identical experiments were performed with glomeruli isolated from rats depleted of bone marrow-derived phagocytes by irradiation (Figure 4). There was no significant difference in TNFα production by these glomeruli compared with that by normal controls. Thus, infiltration of glomeruli by tissue macrophages does not account for the observed results.

**Figure 2.** Dose response of LPS-induced TNFα production by isolated glomeruli. Kidneys were removed 1 h after the iv injection of various doses of LPS (from 10 to 500 μg/kg), and isolated glomeruli were incubated at 37°C for 4 h. Supernatants were assayed for the presence of TNFα by bioassay. Results represented mean ± SE of four experiments. ANOVA indicated that TNFα production varied significantly depending upon LPS dose (P < 0.005). BW, body weight.

**Characterization of Interleukin Production by Glomeruli During Experimental Endotoxemia**

IL-1 bioactivity, determined by the standard thymocyte assay, was not detectable in cell lysates and supernatants of glomeruli isolated at any time after *in vivo* LPS challenge. By contrast, supernatants obtained from peritoneal macrophages cultured in the presence of 1 μg/mL of LPS for 18 h contained 16 to 32 U/mL of bioactive IL-1.

IL-6 bioactivity, determined by the standard B9 cell proliferation assay, was detectable in supernatants of glomeruli isolated within 1 to 2 h after LPS injection (Figure 5). We next designed a series of experiments to evaluate the interactions between TNFα and IL-6. The kinetics of TNFα and IL-6 production by glomeruli isolated from control rats and exposed *in vitro* to LPS are depicted in Figure 6A. The IL-6 appearance was associated with the termination of TNFα production, suggesting that IL-6 could participate in a negative feedback loop blocking TNFα production. However, this is unlikely because neutralization of IL-6 activity by a specific antibody only slightly increased LPS-induced TNFα production (P < 0.05) (Figure 6B), and conversely, the addition of recombinant mouse IL-6 at concentrations of up to 150,000 U/mL did not reduce LPS-induced TNFα production.
production (46.4 ± 18.2 and 37.5 ± 8.4% cytotoxic activity in the supernatants of glomeruli exposed for 2 h to 10 μg/mL of LPS together with or without IL-6, respectively; N = 3).

Additionally, the sequential appearance of TNFα and IL-6 suggested a role for TNFα as a positive regulator of IL-6 levels. However, the pretreatment of glomeruli with an anti-mouse recombinant TNFα polyclonal antibody that suppressed TNFα bioactivity did not affect IL-6 production (Figure 6C).

DISCUSSION

Endotoxemic shock is a significant cause of morbidity and mortality in hospitalized patients. Many of the acute effects of endotoxemia are due to aberrant production of cytokines, especially TNFα (8), resulting in particular in renal failure (22). Previous studies have demonstrated that serum levels of TNFα are effectively elevated after administration of LPS (12). However, because TNFα is essentially an autacoid acting near its site of production, we thought it of interest to evaluate TNFα production in glomeruli.

In our study, we showed that glomeruli isolated from control rats produced low amounts of bioactive TNFα (Figure 1). These results agree with those of previous studies that demonstrate a weak production of TNFα by glomeruli isolated from normal rabbits (23). A striking augmentation of TNFα bioactivity was observed in glomeruli within 1 h after LPS injection in the rat. This TNFα production, which was demonstrated by both a standard L-929 cell cytotoxic
Reciprocal regulatory effects of TNFα and IL-6 on their in vitro synthesis by isolated glomeruli. (A) Time course of TNFα and IL-6 production by isolated glomeruli. Glomeruli isolated from control rat kidneys were incubated at 37°C with 10 μg/mL of LPS for various periods of time. Supernatants were assayed for the presence of TNFα (closed box) and IL-6 (open box) by L-929 fibroblast lytic assay and B9 assay, respectively. Results represented mean ± SE of three experiments. (B) Effect of anti-TNFα and anti-IL-6 antibodies on TNFα production by isolated glomeruli. Glomeruli isolated from control rat kidneys were incubated at 37°C for 4 h with 10 μg/mL of LPS together with or without the indicated antibody. Supernatants were assayed for the presence of TNFα by L-929 fibroblast lytic assay. Results represented mean ± SE of three experiments. * P < 0.05, and ** P < 0.005 as compared with the control without antibody (unpaired t test). (C) Effect of anti-TNFα and anti-IL-6 antibodies on IL-6 production by isolated glomeruli. The experimental design is the same as that shown in Panel B. Supernatants were assayed for the presence of IL-6 by B9 assay. Results represented mean ± SE of three experiments.
et al. (4) previously demonstrated that, at least at 50 min post-endotoxin administration, the cortical tissue was free of cellular infiltrates. Moreover, there was no significant difference in TNFα production by glomeruli isolated from macrophage-depleted rats and control rats (Figure 4). Thus, the glomerular infiltration by macrophages probably does not account for the observed results. Because rat mesangial cells in culture have been shown to produce TNFα upon LPS challenge (15, 16), TNFα originated more likely from resident cells.

In vivo studies have delineated a variety of biologic effects of TNFα of potential relevance to LPS-induced glomerular injury (31). For example, TNFα appears to play a role in the decrease of GFR by inducing the synthesis of eicosanoids (11) and PAF (9). The latter agent is responsible for changes in cytoskeletal organization compatible with mesangial cell contraction. The other major effects of TNFα on mesangial cells are to increase the release of tissue factor-like procoagulant activity that induces fibrin formation (32) and to up-regulate the mesangial expression of intercellular adhesion molecule-1 (33), IL-8 (34), and colony-stimulating factors (35) that provoke the local adhesion and activation of granulocytes or monocytes. In turn, these blood-borne cells could contribute to the generation of factors that affect the structure and the function of glomeruli. Thus, these observations indicate that LPS has direct effects on the release of TNFα from glomerular cells (Figure 5), but also suggest that the damaging effect of locally produced TNFα may require the participation of circulating cells. This conclusion is in accordance with the observations by Cohen et al. (36). The authors showed that in the isolated rat kidney perfused with a cell-free perfusate, LPS had only a weak effect on renal vascular resistance, whereas the administration of LPS in vivo had profound renal hemodynamic effects.

The sequential release of TNFα, IL-1, and then IL-6 has been demonstrated in the serum of rats given E. coli infusion in vivo (37). By contrast, no detectable IL-1 bioactivity was found in the supernatant or in the cell lysate of glomeruli at any time after LPS infusion (Figure 5). This is consistent with the observation that the extracellular and cell-associated levels of IL-1 are barely detectable in mononuclear cells (38) or in isolated glomeruli (39) after 2 h of incubation with LPS. The fact that RBF falls to its lowest level within 35 min after the administration of LPS (4) suggests that IL-1, via its effects on eicosanoid synthesis (40), does not contribute to the renal hemodynamic changes of experimental endotoxemia.

Of interest, glomerular IL-6 production was induced after LPS challenge both in vivo (Figure 5) and in vitro (Figure 6A). The delay observed between TNFα and IL-6 production raised the possibility that TNFα might induce this release of IL-6, as previously described in mesangial cells (35). However, this is unlikely because the addition of anti-TNFα antibody was not able to attenuate IL-6 response, whereas it almost completely blocked TNFα bioactivity (Figure 6). Elucidation of the role of glomerular IL-6 needs future investigations. In vitro studies have established that besides its well-demonstrated role of growth factor for mesangial cells (41, 42), IL-6 could induce the adherence of circulating cells to the glomerular capillary wall (43). In addition, after release into the circulation, IL-6 could contribute to enhance the production of hepatic acute-phase proteins and to promote fever (44).

In conclusion, this study provides evidence that specific cytokines, namely TNFα and IL-6, are produced within rat glomeruli in response to LPS. The time course of production demonstrated in this model of endotoxin-induced acute renal failure supports the role of TNFα in the induction of early mediators—eicosanoids and PAF—responsible for renal hemodynamic changes.

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"Study with me, then, a few things in the spirit of truth alone, so that we may establish the manner of Nature's operations .... For this essay which I plan will shed light upon the structure of the kidney. Do not stop to question whether these ideas are new or old, but ask, more properly, whether they harmonize with Nature. And be assured of this one thing, that I never reached my idea of the structure of the kidney by the aid of books, but by the long, patient and varied use of the microscope. I have gotten the rest by the deductions of reason, slowly, and with an open mind, as is my custom."